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THE ROLE OF LOCALLY-PRODUCED GROWTH FACTORS ON HUMAN PLACENTAL GROWTH AND INVASION

By

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Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
Canada
April 1994

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ISBN 0-315-93181-7

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ABSTRACT

Trophoblast cells are fetally-derived cells in the placenta which actively invade the uterus during pregnancy to tap into the maternal vessels in the uterus. Trophoblast proliferation, differentiation, and invasion are key events during normal human placental growth and development. These processes must be strictly controlled both temporally and spatially. Studies presented in this thesis define the roles of some locally produced growth factors on trophoblast growth and invasion. The growth factors examined were three ligands of the epidermal growth factor (EGF) receptor: EGF, transforming growth factor (TGF)α, and amphiregulin (AR), as well as colony stimulating factor (CSF)-1, TGFß and insulin-like growth factor (IGF)-II.

Previous studies from this and other laboratories have established the presence and location of EGF, TGFα, CSF-1, and IGF-II in the human placenta and decidua at various gestational ages. The present study examined the distribution of immunoreactive AR, TGFß, and its natural inhibitor decorin in the human gestational tissues throughout pregnancy. AR exhibited a unique spatial and temporal distribution, being present only in the syncytiotrophoblast cell layer of the placenta until approximately week 18 of gestation, and never in the decidua. TGFß was present in the decidua as well as the villous syncytiotrophoblast and extravillous cytotrophoblast cells throughout gestation. Decorin immunoreactivity displayed a pattern of distribution similar to the distribution of TGFß in first trimester decidual tissue, suggesting that decorin may influence TGFß activity in first trimester decidua.

In order to investigate the effects of the growth factors on trophoblast cell growth

and invasion, first trimester human trophoblast cells were isolated from chorionic villus explant cultures, propagated in vitro (2-4 passages) and phenotyped at the light and electron microscope (EM) levels. EM immunogold labeling of cells from explants grown on type IV collagen gel revealed two trophoblast cell populations containing cytokeratin: invasive mononucleate cells which produce fibronectin and oncofetal fibronectin, and noninvasive multinucleate cells which contain HPL. Early passage trophoblast cells were positive for cytokeratin (100%), NDOG5 (50%; an intermediate trophoblast cell marker), PCNA (small proportion), and hPL (multinucleate cells only). These cells were utilized to examine the effects of the growth factors on their proliferative ability (^{3}H-TdR incorporation) as well as the ability to invade an artificially reconstituted basement membrane (matrigel) in vitro. Expression of invasion regulating molecules (type IV collagenase and TIMP's) was examined with Northern analysis and protein zymography.

Functional studies revealed that exogenous EGF, TGF-α, and AR were able to stimulate trophoblast proliferation in a concentration-dependent manner. EGF and TGFα however did not influence trophoblast invasion even though an increase in the mRNA and protein levels of invasion regulating molecules (72 and 92 kDa type IV collagenases and TIMP-1 and TIMP-2) were noted. While exogenous CSF-1 had no effect on trophoblast proliferation or invasion, endogenous CSF-1 appeared to upregulate proliferation as noted from the antiproliferative action of a neutralizing antibody. Exogenous IGF-II demonstrated no significant effect on trophoblast proliferation; however, it significantly enhanced the invasiveness of first trimester human trophoblast cells in a concentration-dependent manner. This stimulation was further enhanced in a synergistic manner in the presence of IGF binding protein (IGFBP)-1.

Choriocarcinomas cell lines (JAR and JEG3) were generally unresponsive to the addition of growth factors (TGFα, EGF, CSF-1) with the exception of the invasion promoting effects of TGFα noted on JAR cells.

The above results suggest that: (a) Both autocrine and paracrine derived growth factors such as EGF receptor ligands and CSF-1 are required for trophoblast growth but not invasiveness, whereas, trophoblast derived IGF-II as well as decidua derived IGFBP-1 interact in promoting trophoblast invasion. (b) TGFß (having earlier shown to be antiproliferative and anti-invasive for trophoblast cells) produced by the decidual cells may be bound to decorin in the decidual ECM. Whether this binding inactivates TGFß in situ remains to be tested. (c) Trophoblast proliferation and invasion are independent functions which can be differentially regulated by local growth factors. (d) These regulatory mechanisms appear to be deranged in certain choriocarcinomas.

These studies are pertinent to a better understanding of implantation and placentation in the human, and pathophysiology of pregnancy associated diseases such as pre-eclampsia (in which trophoblast cells are hypo-invasive), as well as trophoblast malignancies (choriocarcinomas).

To my wife Renée Ann

You're in my heart
You're in my soul
You'll be my breath should I grow old
You are my lover
You're my best friend
You're in my soul

ACKNOWLEDGEMENTS

I thank my supervisor, Dr. P.K. Lala, for providing me the opportunity to work in his laboratory. His support, encouragement, and enthusiasm for science made his laboratory an excellent place to develop and grow into a scientist.

I am also very grateful to everyone in Dr. Lala's lab for their continued support and friendship throughout the years. I thank Nelson Khoo for his constant friendship and for his expertise in molecular biology. I am thankful to Julie Irving, Amila Orucevic, Mary Nel Saarloos, Nahla Al-Mutter, Ian Connelly, and Anne Pin for their continuous support and assistance on numerous occasions. I would also like to thank Dr. Charles Graham for collaborating with me in a number of experiments and for his friendship.

I am thankful to all the members of the Department of Anatomy for making my stay here very enjoyable and stimulating.

I would like to thank my collaborators on a number of projects these include; Dr. Victor Han for the insulin-like growth factor-II studies, Dr. Gibbs Johnson from the NIH, for the amphiregulin studies, Dr. Gordon Pringle for the decorin studies, and Steve Hearn for the electron microscopic studies.

I am grateful to the members of my advisory committee, Drs. Peter Merrifield and Tom Kennedy for reading this thesis and providing with me with ideas to improve upon it.

I would like to thank my parents and sister for their constant love and support throughout my academic career. They have always been there when I needed them and always provided encouragement.

Finally, I thank my best friend, my wife Renée, if it was not for her endless encouragement and love, none of this work would have been possible.

Too many times we stand aside
And let the water slip away
'Til what we put off 'til tomorrow
Has now become today
So don't you sit upon the shoreline
And say you're satisfied
Choose to chance the rapids
And dare to dance the tide.

Garth Brooks

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LIST OF ABREVIATIONS

ABC -	avidin biotin complex
Abs -	antibodies
AR -	amphiregulin
α_2M -	α_2-macroglobulin
BP -	binding protein
BSA -	bovine serum albumin
CM -	complete media
cDNA -	copy DNA
CSF -	colony stimulating factor
DAB -	diaminobenzidene
DEPC -	diethyl pyrocarbonate
DNA -	deoxyribonucleic acid
dpm - per	decompositions minute
ECM -	extracellular matrix
EDTA -	ethylenediamine-tetracetic acid
EGF -	epidermal growth factor
EGFr -	epidermal growth factor receptor
EtOH -	ethanol
hCG -	human chorionic gonadotropin
Ig -	immunoglobulin
IGF -	insulin-like growth factor
IGFBP -	insulin-like growth factor binding protein
IL -	interleukin
MMP -	matrix metalloprotease
O.D. -	optical density
PA -	plasminogen activator
PAI -	plasminogen activator inhibitor
PBS -	phosphate buffered saline
PCNA -	proliferating nuclear antigen
PCR -	polymerase chain reaction
PG -	prostaglandin
RER -	rough endoplasmic reticulum
RNA -	ribonucleic acid

RT-PCR - reverse transcriptase polymerase chain reaction

SDS - sodium dodecyl suphate

SFM - serum free media

SRM - serum reduced media

SSC - standard saline citrate

TdR - thymidine

TGF - transforming growth factor

TIMP - tissue inhibitor of metalloproteinase

tPA - tissue plasminogen activator

uPA - urokinase plasminogen activator

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I. INTRODUCTION

The human placenta is a multifunctional organ essential for the development of the embryo. It is a unique structure sharing dual properties of an allograft as well as a locally invasive tumor (reviewed by Lala, 1990). It is comprised primarily of fetally-derived cells which are genetically disparate from the mother, and is yet unharmed by the maternal immune system. It is a highly invasive structure, and yet does not destroy the uterus. Mechanisms responsible for uteroplacental homeostasis have been the subject of investigation from this laboratory for one and a half decades.

The cellular architecture of the human placenta is obtained by the ability of fetally-derived trophoblast cells to rapidly proliferate, invade into the endometrium and tap into uterine blood vessels. These normal physiological functions of trophoblast cells have many similarities with those of malignant tumor cells. In both cases rapidly growing cells are capable of invading and migrating into normal tissue spaces and blood vessels. However, unlike the uncontrolled growth and invasion by malignant tumor cells, trophoblast cell growth and invasion are highly regulated events restricted both spatially and temporally.

The factors governing trophoblast cell growth and invasion have been under investigation for many years. Three decades ago Kirby (1960; 1963a; 1963b; 1965) examined the extent of tissue invasion by the murine trophoblast transplanted into ectopic and orthotopic sites and concluded that the decidual tissue of the uterus may control trophoblast invasiveness by "mechanical" or "chemical" means. However, the "means" remained unidentified because of Kirby's untimely death, until the late 1980s when Dr. P.K. Lala's laboratory began to investigate the questions originally raised by Kirby.

Work from this laboratory revealed that human trophoblast cells employ mechanisms identical to those executed by highly invasive tumor cells to invade natural or artificially reconstituted basement membranes (Yagel et al., 1988; Yagel et al., 1990; Lala and Graham 1990; Graham and Lala, 1991; Graham and Lala 1992); however, trophoblast invasion of the uterus is controlled by local factors, in particular, decidua-derived transforming growth factor (TGF)-β (Graham et al., 1992).

Over the past decade the role of growth factors in normal physiological and pathological events has been the topic of extensive research. Growth factors have been identified in virtually all tissues and new growth factors are being discovered at a rapid rate. In the present study, the effects of a number of locally-produced growth factors on normal first trimester human trophoblast cell growth and invasion were examined. These studies are relevant to an understanding of normal placental development and may provide insight into pathological conditions, for example, preeclampsia (associated with poor placental invasion), intrauterine growth retardation (associated with poor placental growth), and choriocarcinoma (associated with extensive trophoblast growth and invasion).

II. HISTORICAL REVIEW

1. THE HUMAN FETOMATERNAL INTERFACE

Anatomically, the human fetomaternal interface consists of the placenta, a fetally-derived organ and the decidua, a maternally-derived tissue. Physical as well as molecular interactions at this interface hold the secrets to two important biological riddles: (1) what protects the placenta, a fetally-derived organ and thus genetically disparate from the mother, from destruction by the mother's immune system? (2) what protects the uterus from overinvasion by the placenta which is a highly invasive tumor-like structure? This thesis will focus on studies of locally-derived growth factors and their effects on trophoblast growth and invasion.

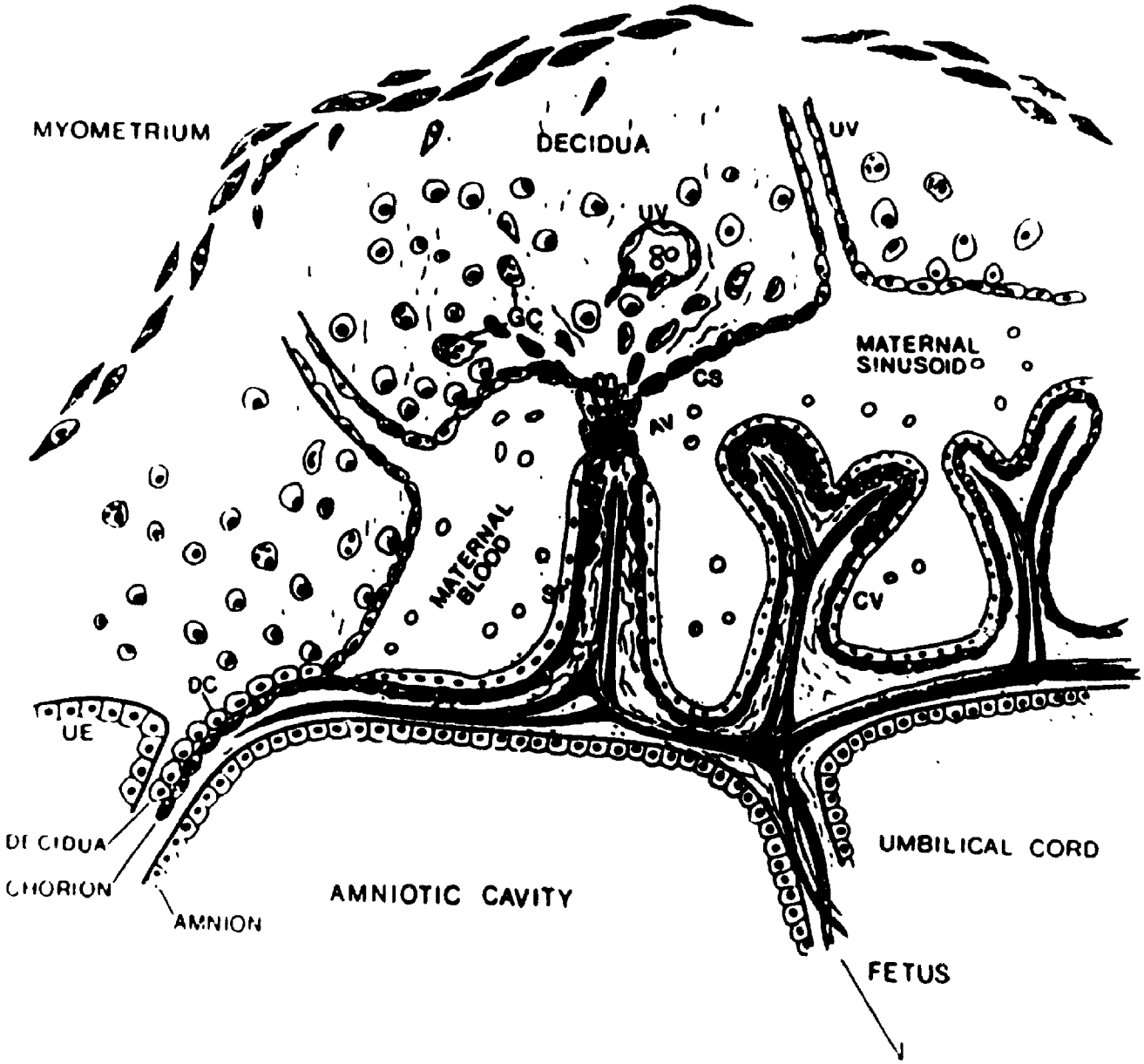
1.1. The Placenta

The placenta is an organ essential for the proper development of the embryo or the fetus. The architecture of the placenta brings maternal and fetal blood in close proximity with each other to allow for an efficient exchange of molecules. Nutrients and oxygen are transferred from the maternal to the fetal blood, while fetal waste products are passed from the fetus to the mother. The placenta is also the largest endocrine organ secreting numerous steroid and protein hormones, as well as the source of a large number of locally active growth factors essential for the maintenance of pregnancy.

The structure of the human placenta is obtained by the ability of trophoblast cells to rapidly proliferate, migrate, and invade into the endometrium inclusive of capillaries

and glands. and finally tap into the spiral (uteroplacental) arteries of the uterus. In humans, placental development begins approximately six days after fertilization as the blastocyst attaches to the apical surface of the uterine epithelium usually on the posterior wall of the uterus (Hertig and Rock, 1945). In subsequent steps, the blastocyst breaches the epithelial barrier and trophoblast cells begin to invade the stroma in the endometrial lining of the uterus. The integrity of the uterine epithelium is soon re-established, thus positioning the blastocyst completely in the endometrial stroma. As implantation proceeds, trophoblast cells continue to invade endometrial capillaries, glands, and the decidua. Invasion of capillaries creates sinusoidal spaces surrounding villous projections of the trophoblast (primary villi) consisting of an inner layer of cytotrophoblast cells and an outer syncytiotrophoblast layer. Soon secondary villi form after extraembryonic mesoderm penetrates the villus core. Shortly thereafter, fetal vessels develop in the mesodermal core of the villi transforming them into tertiary villi which grow and branch, forming tree-like structures bathed in the maternal blood known as "floating" chorionic villi (Figure 1). In some areas cytotrophoblast cells sprout from the tips of the chorionic villi (anchoring villi) through the syncytial layer to extend as columns invading the maternal decidual tissue. This trophoblast cell population is commonly described as the intermediate trophoblast. Within the decidual tissue some of these cells form the cytotrophoblastic shell which anchors the placenta to the uterus (Boyd and Hamilton, 1970) and others remain isolated or fuse to form placental bed giant cells. Yet other intermediate trophoblast cells seek out spiral arteries of the uterus, erode their tunica media, replace their endothelium, and prevent them from responding to vasoactive substances in the maternal blood. These cells are often described as the endovascular

Figure 1: Schematic diagram demonstrating the histology and relationships of the placenta and the uterus at approximately the end of the first trimester in the human. Anchoring villi (AV), serve to attach the placenta to the uterine wall. Cells migrating out from anchoring villi, extravillous trophoblast (ET) cells form the cytotrophoblastic shell (CS) or remain embedded in the decidual tissue as isolated cells. These isolated ET cells are highly invasive, invading as far as the myometrium or invading maternal blood vessels. When ET cells differentiate they fuse and form placental bed giant cells (GC), which are presumably noninvasive. CV, chorionic villus; CT, cytotrophoblast; ST, syncytiotrophoblast; DC, decidual cell; UE, uterine epithelium, UV, uterine blood vessel. With permission from Graham and Lala, 1992.


MYOMETRIUM
DECIDUA
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MATERNAL SINUSOID
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AV
MATERNAL BLOOD
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DC
UE
DECIDUA
CHORION
AMNION
AMNIOTIC CAVITY
UMBILICAL CORD
FETUS

trophoblast. This process is essential for maintaining an adequate perfusion of maternal blood to the placenta (Loke, 1990). A schematic diagram of the human placenta inclusive of various trophoblast cell populations is presented in figure 1.

Many similarities are shared between the placenta and invasive tumors (Lala, 1990; Lala and Graham, 1990; Graham et al., 1992; Graham and Lala, 1992). In both situations rapidly growing cells are capable of transgressing normal tissue barriers and invading blood vessels. The formation of new blood vessels, angiogenesis, is also common to both the placenta and malignant tumors. However, unlike the uncontrolled growth and invasion by cancer cells, trophoblast cell growth and invasion are highly regulated events restricted both spatially and temporally. This thesis will describe the roles of locally-produced growth factors regulating these events.

1.2. The Decidua

The decidua is a maternally-derived tissue comprising decidual cells, lymphocytes, monocytes, macrophages, granulocytes, stromal fibroblasts, and blood vessels (Kearns and Lala, 1985; Peel, 1989). Decidual cells represent a distinct cell class which arises in the endometrium during pregnancy through the proliferation and differentiation of endometrial stromal cells (Zhinkin and Samoskina, 1967; Galassi, 1968; Das and Martin, 1978). The formation of the decidua (decidualization) during pregnancy succeeds the implantation of the blastocyst. However, in nonpregnant women, a small amount of decidualization may occur in the late secretory phase of the menstrual cycle (Bell, 1983). In the human, the decidua can be subdivided into three distinct zones: the decidua

basalis, in the endometrium directly apposed to the villus trophoblast of the placenta; the decidua capsularis, in the endometrium adjacent to the amnio-chorion (nonvillous trophoblast); and the decidua parietalis, in the remaining endometrium of the uterus facing the lumen. While it is unclear at present, whether these geographically distinct zones are also distinct functionally, most functional studies have been conducted with decidua basalis. In rodents (mouse and rat) the decidua initially appearing at the implantation site (antimesometrial decidua) exhibits some morphological and phenotypic differences from the later appearing decidua in contact with the developing placenta (mesometrial decidua). Antimesometrial decidua is more compact, composed of larger, highly polyploid decidual cells (O'Shea, 1983) which express insulin-like growth factor binding protein (IGFBP) - 1 (Dr. V.K.M. Han, personal communication) and decidual prolactin related peptide (Gu et al., 1992). Mesometrial decidua is more loose, composed of smaller decidual cells (O'Shea, 1983), which express α_2 macroglobulin (Gu et al., 1992) and prolactin receptors (Gu et al., 1992).

The life history of decidual cells has been studied extensively in the murine and rat models. It has been shown that decidual cells arise from the proliferation and differentiation of their immediate precursors, the uterine stromal fibroblast-like cells (Galassi, 1968; Das and Martin, 1978). Because of the local immunoregulatory role of decidual cells in the uterus, Kearns and Lala (1982) hypothesized that predecidual stem cells may originate from the bone marrow. They addressed this question in the mouse by making radiation bone marrow chimeras and then screening single cell suspensions of deciduoma (decidualization produced in pseudopregnant animals) for the presence of donor derived cells of disparate H-2 phenotype. They found that at least a subpopulation

of decidual cells was derived from ultimate progenitors present in the bone marrow. Johnson et al (1989) confirmed these findings for the decidua of normal murine pregnancy by transplanting H-2 disparate donor hemopoietic cells into the yolk sac of developing embryos to produce fertile bone marrow chimeras. By taking advantage of this technique developed in this laboratory by Johnson et al (1989) to make prenatal chimeras which retained fertility, but employing donor bone marrow cells bearing a transgenic marker (1000 copies of ß-globin gene), Lysiak and Lala (1992) recently identified and characterized in situ the decidual cells of normal murine pregnancy, having a hemopoietic descendence. These experiments in mice clearly reveal that predecidual stem cells, at least those destined for the decidua basalis (mesometrial decidua), are hemopoietic in origin and migrate to the uterus at some point in ontogeny. Whether this is also the case in the human remains to be determined.

The decidua has been shown to play an important role in the maintenance of pregnancy. Decidual cells in the human produce prolactin (Riddick and Kusmik, 1976; Kubota et al.,1981) as well as short range biological mediators eg growth factors (Graham et al.,1992) and prostaglandins of the E (PGE) series (Parhar et al.,1988). Decidual cells in the rat make prolactin-like peptides (Gu et al., 1992), as well as short range mediators eg growth factors such as transforming growth factor (TGF)α (Han et al., 1987; Bonvissuto et al., 1992) and PGE_2 (Kennedy, 1979, 1980a, 1980b). In both the human and the mouse, the decidua has been reported to exert a local immunoprotective function for the conceptus through the release of PGE_2 (Lala et al.,1986, 1988; Scodras et al.,1990; Parhar et al.,1988; Parhar et al.,1989; Tawfik et al.,1986; Mathews and Searle, 1987). Decidua-derived PGE_2 has been found to

inactivate T cells (Parhar et al.,1988; Parhar et al.,1989) as well as natural killer (NK) cells (Parhar et al.,1989; Scodras et al.,1990) in situ in the human and the murine decidua. This inactivation results, at least in part, from a down regulation of interleukin (IL)-2 receptors on effector cells and an inhibition of IL-2 production (Lala et al.,1988; Parhar et al.,1989), mechanisms which would prevent accidental activation of decidual lymphocytes into lymphokine activated killer (LAK) cells which express trophoblast killer ability (Parhar et al.,1989). TGFß$_2$-like molecules produced by the murine decidua has also been reported to have an immunoprotective role (Clark et al.,1990).

Recent studies from this laboratory have shown that the decidua also plays a major role in the regulation of trophoblast growth (Graham et al.,1992) and invasion (Graham, Lala, 1991; Graham, Lala, 1992) by secreting a number of growth factors. These studies will be described and discussed later.

2. GROWTH FACTORS

A large number of growth factors have been reported to be produced by the human placenta and/or the decidua, which may be functionally important for the conceptus. The present study has focused on some of these growth factors relative to their role on trophoblast proliferation, differentiation, and invasion. They include epidermal growth factor (EGF), transforming growth factor (TGF)-α, amphiregulin (AR), all binding to the EGF receptor, as well as TGFß, insulin-like growth factor (IGF)-II, and colony stimulating factor (CSF)-1. These growth factors have been demonstrated in the human gestational tissues and thus may influence trophoblast function.

2.1. Epidermal Growth Factor Receptor Ligands

Proteins belonging to the EGF family all have a common consensus sequence of $CX_7CX_{4-5}CX_{10-13}CXCX_8C$ contained within 36-40 amino acid residues and are synthesized as larger membrane-bound, glycosylated precursors (reviewed by Prigent and Lemoine, 1992). Members of this family have been shown to be powerful mitogens for a variety of epithelial cells. They exert their mitogenic actions through binding to the EGF receptor (EGFr), which possesses tyrosine kinase activity (reviewed by Carpenter and Wahl, 1991).

2.1.1. Epidermal Growth Factor

Initially isolated from mouse maxillary glands and originally termed "tooth-lid factor" because of its ability to cause precocious eyelid opening, and incisor eruption in mice (Cohen, 1962), this factor was soon renamed as epidermal growth factor for its effects on epidermal growth (Cohen et al., 1964). Mature human EGF is a 53 amino acid polypeptide (6 kDa) derived by cleavage of a large 1217 amino acid (130 kDa) transmembrane precursor molecule (Bell et al.,1986; reviewed by Carpenter and Wahl, 1991). Studies using several mouse tissues, particularly distal renal tubule cells, have found that some cells do not process the EGF precursor molecule suggesting that the precursor may be a cell surface protein involved in some recognition function (Rall et al., 1985). Recent experiments using an EGF mini-gene expression system have demonstrated that membrane-bound EGF was capable of binding to EGF-receptors on

adjacent cells and exerting a biological response (Dobashi and Stern, 1991), the so called "juxtacrine" function. EGF has a broad distribution in the body and has been implicated in many normal physiological events including wound healing (Ross et al., 1986), eyelid opening and tooth eruption (Cohen, 1962), bone resorption (Raisz et al., 1980), ulcer prevention (Sakamoto et al., 1985), increasing T cell proliferation (Acres et al., 1985), neuron survival and process outgrowth (Morrison et al., 1987) and most importantly, epithelial cell proliferation during normal renewal (reviewed by Carpenter and Wahl, 1991).

2.1.2. Transforming Growth Factor α

TGFα was initially termed sarcoma growth factor (SGF) from its ability to reversibly transform certain cells and to compete with EGF for binding to the EGF-receptor (Todaro and De Larco., 1976). SGF was later found to consist of two distinct factors, TGFα being the EGF-related factor and TGFß being the other factor (Anzano et al., 1983). TGFα is 50 amino acid polypeptide derived from a larger precursor molecule, pro-TGFα (Lee et al., 1985). It has been found to exist in several molecular weight forms ranging from 5 to 20 kDa depending upon its extent of glycosylation and proteolytic cleavage (Bringman et al., 1987). At the amino acid level there is 90% homology among mouse, rat, and human TGFα. TGFα belongs to the family of EGF-related proteins exhibiting, in the human, 40% homology with EGF (reviewed by Salomon et al., 1990).

Pro-TGFα is a membrane bound glycoprotein of 160 amino acids (Derynck et al.,

1984). Proteolytic cleavage of pro-TGFα is required for the release of mature TGFα from the cell membrane (Bringman et al., 1987; Brachman et al., 1989; Teixido et al., 1990). It has been demonstrated using mutated noncleavable forms of pro-TGFα that it is capable of exerting biological activity (Wong et al., 1989; Brachman et al., 1989). Thus, if two cells are in close apposition to one another, with one expressing pro-TGFα and the other the EGFr a juxtacrine type of stimulation may occur (Anklesaria et al., 1990). Proteolytic cleavage of pro-TGFα may act as a regulatory step between membrane-bound pro-TGFα and diffusible TGFα, but is not essential for its biological activity (reviewed by Massague, 1990).

Originally believed to be involved in carcinogenesis, TGFα is now known to be synthesized by many normal cells types and implicated in several normal physiological events such as tooth eruption (Tam et al., 1985), eyelid opening (Smith et al., 1985), inflammation (Schultz et al., 1987), cell migration (Barrandon and Green, 1987), bone resorption (Stern et al., 1985), and angiogenesis (Schreiber et al., 1986). TGFα expression has also been noted in many types of human cancers (reviewed by Salomon et al., 1990; reviewed by Prignet and Lemoine, 1992). Transgenic mice which over express TGFα have been reported to exhibit disordered growth and differentiation in the mammary gland, liver, and pancreas and ultimately breast and liver neoplasia (Sandgren et al., 1990; Jhappan et al., 1990; Matsui et al., 1990). Recent work by Luetteke et al (1993) using homologous recombination in embryonic stem cells to disrupt the TGFα gene in mice revealed that mice which completely lacked functional TGFα gene survived the embryonic/fetal periods but exhibited a wavy coat pattern with abnormal hair follicle orientation, and some mice developed eye malformations, suggesting that other EGF-

receptor ligands assumed the role of TGFα in critical developmental events.

2.1.3. Amphiregulin

AR is an 84 amino acid glycoprotein originally purified from the conditioned media of a human breast cancer cell line treated with 12-*O*-tetradecanoylphorbol-13-acetate (Shoyab et al.,1988). Like TGFα, the mature AR peptide is believed to be the proteolytic cleavage product of a 252 amino acid transmembrane precursor (Plowman et al.,1990). The COOH-terminal of AR shares 38% homology with EGF and 32% homology with TGFα (Shoyab et al.,1989). The biological activity of AR is believed to be mediated through the EGF receptor since AR is able to compete with ^{125}I-EGF for binding; however, two putative nuclear targeting sequences have also been described in its NH_2-terminal region (Shoyab et al.,1989) and immunoreactive AR has been found in the nuclei of normal and malignant ovarian and colonic epithelial cells (Johnson et al.,1991; Johnson et al.,1992).

AR is often described as a bifunctional growth-modulating protein because of its ability to either stimulate or inhibit the proliferation of a variety of normal and tumor cell types (Johnson et al.,1992; Johnson et al.,1991; Shoyab et al.,1988). Its affect on proliferation, in certain cell types, was also dependent on the concentration of AR used (Johnson et al.,1991).

2.2. Transforming Growth Factor-ß

As stated earlier, TGFß was isolated as a product of certain sarcoma cells along

with TGFα and both together were termed SGF (Todaro et al., 1976). TGFß is a 25 kDa disulphide-linked homodimer with subunits of 112 amino acids derived from a larger precursor molecule (Derynck et al., 1985). It was originally purified from human platelets (Assoian et al., 1983), human placenta (Frolik et al., 1983), and bovine kidney (Roberts et al., 1983). It is now known that there are five isoforms of TGFß, $TGF\beta_1$ to $TGF\beta_5$, $TGF\beta_1$ being the one first purified. $TGF\beta_1$ - β_3 have been found in mammals and share greater then 70% homology to one another at the amino acid level (reviewed by Lyons and Moses, 1990; reviewed by Roberts and Sporn 1990). $TGF\beta_1$ and β_2 share similar biological activity and are interchangeable in most assays (Seyedin et al., 1987; Cheifetz et al., 1987; Mule et al., 1988). $TGF\beta_4$ was cloned from a chicken chondrocyte library (Jakowlew et al., 1988) and $TGF\beta_5$ was cloned from a frog oocyte library (Kondaiah et al., 1990). All TGFßs show between 60% - 80% homology to one another at the amino acid level (reviewed by Roberts and Sporn, 1990). TGFß belongs to a TGFß supergene family of peptides that shows conservation of positions of seven of the nine cysteine residues. This includes factors such as, mammalian inhibins and activins, Mullerian inhibitory substances, decapentaplegic gene complex (drososphila), Vg1 (amphibian), and bone morphogenetic proteins (BMPs).

TGFß is secreted from most cells in an inactive or latent form (Pircher et al., 1986). The structure of this latent form has been found to consist of a complex of three components: 1) a TGFß binding protein of 125 - 160 kDa which belongs to the EGF gene family; 2) a 40 kDa protein which is the TGFß precursor without the N-terminal signal sequence and the C-terminal mature TGFß; and 3) the dimeric TGFß protein (Miyazono et al., 1988, 1990; Wakefield et al., 1988). Activation of this inactive

complex can be caused by: 1) proteases such as plasmin and cathepsin D (Lyons et al., 1988); 2) endoglycosidase F or sialidase plus sialic acid or mannose-6-phosphate (Miyazono and Heldin, 1989); 3) pH values below 3.5 or above 12.5 (Miyazono et al., 1990). Control of the activation of latent TGFß may be an important step in regulating its biological activity (Wakefield et al., 1987). Thus, even though a cell may secrete latent TGFß and possess TGFß receptors, an autocrine action of TGFß should not be inferred, unless activation of latent TGFß can also be demonstrated (Wakefield et al., 1987). Another possible mechanism for restricting the biological activity of TGFß is the observation that in serum mature TGFß is found associated with α_2-macroglobulin (O'Connor-McCourt and Wakefield, 1987). Its association with α_2-macroglobulin may represent a clearance mechanism for the removal of mature TGFß (Huang et al., 1988; O'Connor-McCourt and Wakefield, 1987). TGFß may also be found associated with matrix proteoglycans, such as decorin. Decorin is a chondroitin-dermatan sulphate proteoglycan which has been shown to limit the biological activity of TGFß (Yamaguchi et al., 1990; Border et al., 1992).

To date, five TGFß binding proteins on the cell surface have been described. They are TGFß receptor types I - IV and endoglin. Type I and type II receptors are both glycoproteins which bind $TGF\beta_1$ with higher affinity than $TGF\beta_2$, and are found in low numbers on the surface of most cells (reviewed by Massagué et al., 1990). The type III receptor or betaglycan is an integral membrane proteoglycan which is present on most cells but not involved in signal transduction (Cheifetz et al., 1987,1988; Ohta et al., 1987). This receptor however, may be involved in presentation of TGFß to the type I receptors, which are known to possess signal transduction properties (Cheifetz et al.,

1988; Ohta et al., 1987; Boyd and Massagué, 1989) or may also act as a reservoir or clearance system for mature TGFß (reviewed by Massagué et al., 1990). Type IV TGFß receptors have been described on GH_3 pituitary cells and their biological significance remain to be determined; however, it is interesting that this binding protein also binds inhibins and activins, the other members of the TGFß family (Cheifetz et al., 1988). Endoglin, a recently described TGFß binding protein which shares amino acid homology with the type III TGFß receptor (betaglycan; Cheifetz et al., 1992) has been localized to the syncytiotrophoblast cell layer of the human term placenta (Gougos et al., 1992). The significance of endoglins on human term syncytiotrophoblast remains to be determined; however, Gougos et al (1992) suggest that: 1) since high levels of endoglins are present on endothelial cells, their presence on syncytiotrophoblast could be ascribed to the endothelial-like characteristics of the syncytium; or 2) endoglins may play a role in adhesion events since endoglins contain an RGD sequence. Earlier studies have shown that in vitro trophoblast syncytium formation is dependent upon the presence of fibronectin and RGD-containing peptides are capable of inhibiting this process (Kao et al., 1988). The possible roles of endoglins as integrin ligands and/or as regulators of adhesion between integrins and their ligands remains to be further investigated.

TGFß has been reported to be implicated in many biological functions for example, myogenesis (Florini et al., 1986; Massagué et al., 1986; Olson et al., 1986), regulation of extracellular matrix components (reviewed by Roberts and Sporn 199:), bone remodelling (Centrella et al., 1988), immunoregulation (Kehrl et al., 1986, 1989), angiogenesis (Roberts et al., 1985; Fiegel and Knighton, 1988), inhibition of intestinal epithelial cell (Kurokowa et al., 1987), bronchial cell (Masui et al., 1986), and

keratinocyte (Moses et al., 1985; Coffey et al., 1988) proliferation, embryogenesis (Akhurst et al., 1990; Pelton et al., 1991), and numerous other functions, including a facilitating role in carcinogenesis (reviewed by Roberts and Sporn, 1991). Using homologous recombination in murine embryonic stem cells, Shull et al (1992) were able to disrupt the TGFß gene. Homozygotes for the mutation exhibited no developmental abnormalities, however, at approximately 20 days postpartum all animals died due to a wasting syndrome with multifocal mixed inflammatory cell response and tissue necrosis. This suggests that TGFß deficiency is associated with severe dysfunction of the immune system, possibly leading to pathological inflammatory responses resulting in death (Shull et al., 1992).

2.3. Insulin-like Growth Factor-II

IGF-II is a 67 amino acid single chain polypeptide which shares homology with IGF-I and human insulin. Unlike IGF-I, IGF-II production is minimally growth hormone dependent (reviewed by Rechler and Nissley, 1991). IGF-II is originally produced as a larger propeptide (Zumstein et al., 1985; Gowan et al., 1987), however, little is known of the processing to the mature peptide (reviewed by Rechler and Nissley, 1991). Results from in situ hybridization studies have revealed high levels of IGF-II RNA in many human fetal tissues such as the liver, skin, adrenal, and skeletal muscles (Han et al., 1988; Gray et al., 1987; Scott et al., 1985). Intermediate levels were found in the pancreas, skin, and the kidney (Han et al., 1988; Scott et al., 1985); whereas, low levels were detected in the heart, intestine, lungs, stomach, and the spleen (Han et al., 1988;

Scott et al., 1985). In many of the fetal tissues examined IGF-II mRNA levels were 100-600 times higher than IGF-I mRNA levels (Han et al., 1988). In adults IGF-II mRNA is also expressed in many tissues, however, at considerably lower levels (Scott et al., 1985; reviewed by Rechler and Nissley, 1991).

The biological effects of both IGF-I and IGF-II are predominantly mediated through the type I IGF receptor which possesses tyrosine kinase activity (reviewed by Rechler and Nissley, 1991). IGF-II is also capable of binding to the IGF-II/mannose-6-phosphate (type 2) receptor; however, its role in the biological actions of IGF-II is still uncertain (Kiess et al.,1988; Mathieu et al.,1990).

The in vivo biological roles of IGF-II are still largely speculative. It is believed to play a role in embryonic and fetal development, particularly in the development of the central nervous system because of its abundance in these structures along with its receptor (reviewed by Rechler and Nissley, 1991). IGF-II is also abundant in human bone matrix. It is synthesized by osteoblasts, stimulates the proliferation of preosteoblasts, and is secreted during bone resorption, thus suggesting a role in bone remodelling (reviewed by Rechler and Nissley, 1991).

In the blood and the extracellular fluid, the IGFs are associated with a family of binding proteins (BPs) termed IGFBPs (reviewed by Rechler and Nissley, 1991). To date, six BPs have been characterized and labeled as IGFBP-1 to -6 (reviewed by Shimasaki and Ling, 1991). The BPs appear to regulate the biological actions of the IGFs by either enhancing or inhibiting their binding to the IGF receptor (DeMellow, Baxter, 1988; Clemmons, 1991; McCusker and Clemmons, 1992). It is interesting to note that BP-1 and BP-2 have an RGD sequence (reviewed by Shimasaki and Ling, 1991) which

can bind to RGD binding sites on the cell surface and thus potentiate binding of certain IGF's to their receptors.

2.4. Colony Stimulating Factor-1

Colony stimulating factor (CSF)-1 is a homodimeric glycoprotein that stimulates the proliferation, differentiation, and survival of monocytes, macrophages, and their committed precursors in the bone marrow (Stanley et al., 1983). In the human, CSF-1 is encoded by a single gene on chromosome 5 consisting of 10 exons (Ladner et al., 1987; Pettenati et al., 1987). Several alternatively spliced mRNAs are transcribed generating both secreted and membrane-bound forms of CSF-1 (Ladner et al., 1987). Two forms of the mature protein are known to exist, a long precursor of 554 amino acids ($CSF\text{-}1^{554}$) which is processed intracellularly to generate a soluble 86 kDa homodimer that is rapidly secreted and a shorter precursor of 256 amino acids ($CSF\text{-}1^{256}$) which exists as a membrane-bound homodimer and cleaved by proteolysis to a 44 kDa molecule (Sherr, 1988). It is believed that both secreted and membrane-bound forms are capable of biological activity and thus CSF-1 may function in an endocrine, paracrine or juxtacrine manner (reviewed by Sherr and Stanley, 1991).

CSF-1 production has been detected in many adult tissues most notably the liver, the placenta, stromal cells of the bone marrow, and numerous fibroblast cell lines (reviewed by Sherr and Stanley, 1991).

CSF-1 exerts its biological actions by binding to the CSF-1 receptor, a receptor with intrinsic tyrosine kinase activity. The CSF-1 receptor is also known as the *c-fms*

protooncogene product. It consists of a 512 amino acid extracellular domain, a 25 amino acid membrane spanning region, and a 435 amino acid tyrosine kinase domain (reviewed by Sherr and Stanley, 1991). Receptor expression is abundant on monocytes, macrophages, and their committed bone-marrow precursors; however, expression on many other cell types have not been excluded (reviewed by Sherr and Stanley, 1991). Indeed, CSF-1 receptors have been detected on human trophoblast cells (Pampfer et al., 1992; Saji et al., 1990) and trophoblast giant cells of the murine placenta (Muller et al., 1983).

3. LOCALIZATION OF GROWTH FACTORS IN THE HUMAN PLACENTA AND THE DECIDUA

3.1. Epidermal Growth Factor and Transforming Growth Factor-α

TGFα was first isolated from acid-ethanol extracts of term human placentas (Stromberg et al.,1982). Haining et al (1991) employed reverse transcriptase-polymerase chain reaction (RT-PCR) to demonstrate the presence of EGF and TGFα mRNA in the human endometrium and term decidua, although the exact cellular source of these peptides remains undetermined. They suggested that these peptides may be required for epithelial regeneration after menstruation and may also be mitogenic for trophoblast growth, since trophoblast cells express EGF-receptors (Lai and Guyda, 1984; Chen et al.,1988; Muhlhauser et al.,1993). Immunohistochemical analysis of EGF in the human placenta and the decidua throughout gestation has localized EGF to uterine epithelial cells, decidual cells, as well as both cytotrophoblast and syncytiotrophoblast (Hofmann

et al.,1991). Recently, mRNA, immunoreactive protein, and biologically active levels of TGFα and EGF were measured in pooled samples of human placenta from early, mid, and late gestations (Bissonnette et al.,1992). These experiments revealed that placentas contained high levels of TGFα and low levels of EGF mRNA as well as proteins throughout gestation. The levels of biologically active EGF-receptor ligands (proteins able to compete with radiolabeled EGF for EGF-receptors) were, however, similar in early and late gestational placentas. These investigators suggested that EGF receptor ligands produced by the placenta, more importantly TGFα and to a lesser extent EGF, may be required for placental development and function (Bissonnette et al.,1992). Filla et al (1993), concurrent with the studies reported in this thesis, have also recently demonstrated the presence of TGFα and the EGF-receptor in the human placenta and the decidua. Using immunohistochemical methods they localized TGFα to cytotrophoblast and maternal decidual cells throughout human gestation; EGF-receptor was present on the villous cytotrophoblasts and the syncytiotrophoblast cell layer. Studies from this laboratory have also immunolocalized TGFα in the human placenta and the decidua throughout gestation (Lysiak et al., 1993). TGFα was found to have a broad distribution, present intracellularly in decidual cells as well as extravillous and villous trophoblast cells throughout pregnancy (Figure 2).

3.2. Transforming Growth Factor-ß

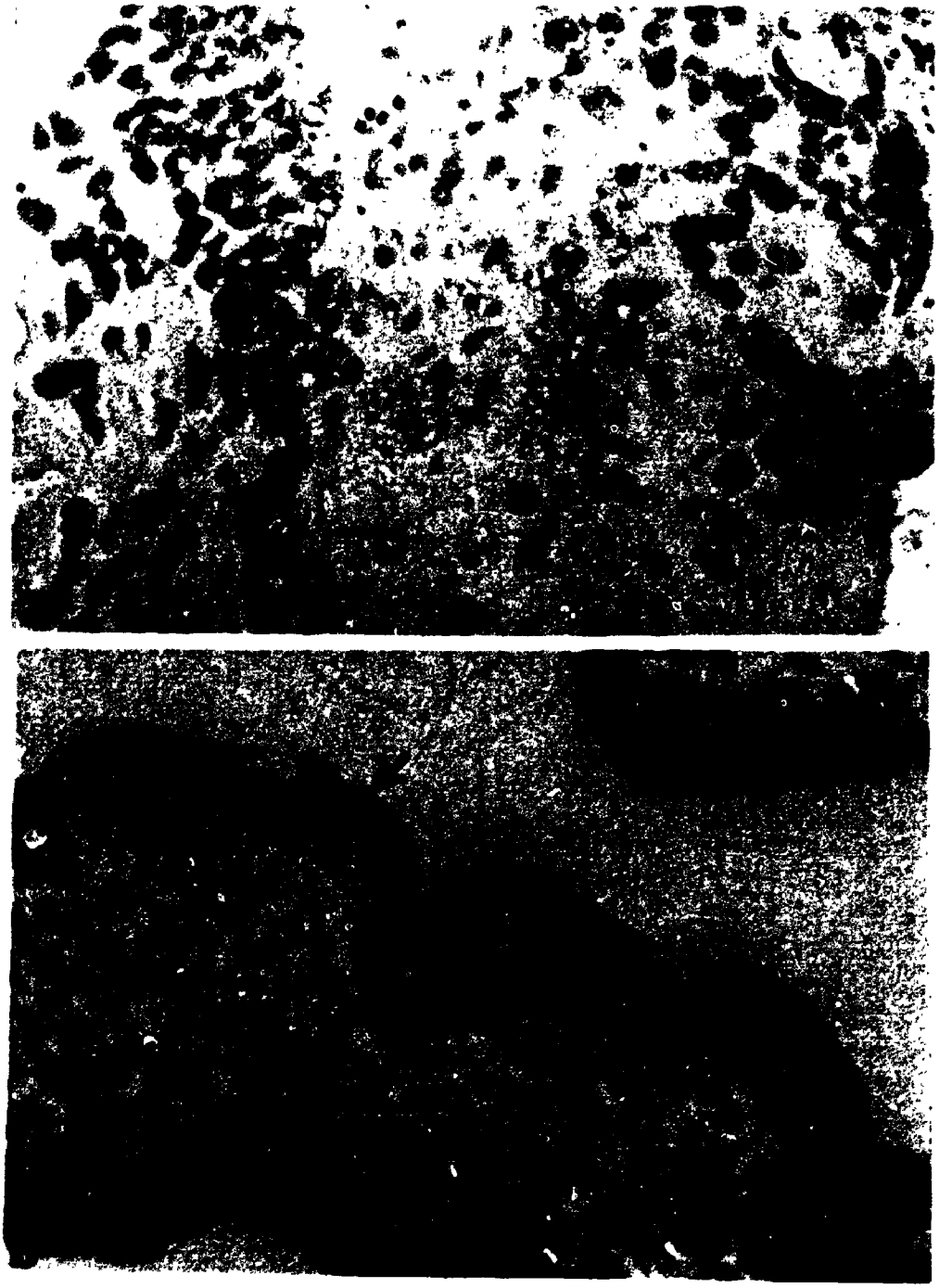
TGFß was originally purified from normal term human placentas (Frolick et al.,1983). TGFß mRNA has been detected in samples of total RNA isolated from human

Figure 2: Photomicrographs of sections of the human decidua and placenta from mid-gestation and the first trimester respectively, immunostained for TGFα.

a) Section of decidua (D) and extravilious trophoblast (ET) cells to demonstrate the intracellular immunostaining of TGFα. X220

b) First trimester chorionic villi immunostained for TGFα revealed immunoreactivity in both the syncytiotrophoblast cell layer (closed arrow) as well as the cytotrophoblast cells (open arrow). X220

Results from the immunolocalization of TGFα in the placenta and the decidua throughout human gestation revealed a broad distribution of the peptide being present in decidual cells as well as villous and extravillous trophoblast throughout pregnancy.



placentas at various stages of gestation with peak expression at 17 and 34 weeks of gestation (Dungy et al.,1991). Using a rabbit polyclonal anti-TGFß antibody, these investigators also found immunoreactive TGFß peptide confined to the syncytiotrophoblast layer of chorionic villi at various gestational ages (Dungy et al.,1991). In situ hybridization studies indicate that syncytiotrophoblast cells also express TGFß mRNA (Hunt, J., personal communication).

Type I, type II as well as type III (betaglycan) receptors for TGFß have been demonstrated in freshly isolated trophoblast cells as well as first trimester trophoblast cells in culture (Mitchell et al.,1992) indicating that locally-derived TGFß may be important for trophoblast function.

3.3. Insulin-like Growth Factor-II

IGF-II has been located in the human placenta and the decidua throughout gestation. Syncytiotrophoblast, cytotrophoblast, intermediate trophoblast, chorion, amnion, and decidual cells all had weak immunoreactivity (Hill et al.,1993). IGF-II mRNA is expressed in situ by the villus cytotrophoblast (Brice et al.,1989) and intermediate trophoblast cells (Han, V.K.M., personal communication) of the human placenta; however, only the latter cells retain this expression throughout gestation, indicating that IGF-II production may be an important function of the invasive trophoblast (Han, V.K.M., personal communication; Figure 3). As mentioned earlier, IGF's may bind to a family of BPs. IGFBP-1 peptide is broadly distributed in the human placenta and the decidua (Hill et al.,1993; Rutanen et al.,1988, 1991; Waites et al.,1989);

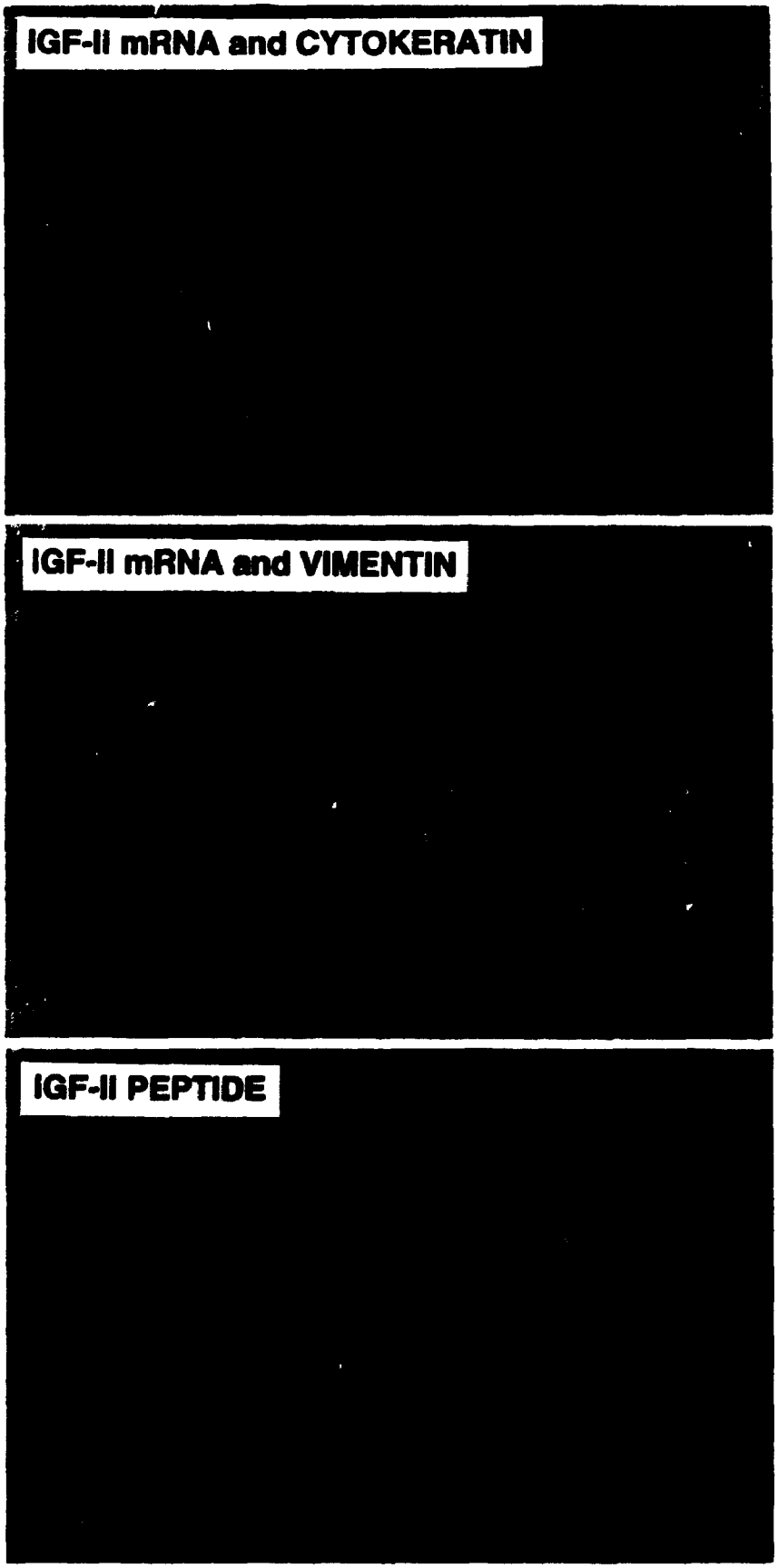
Figure 3: Photomicrographs of sections of the placental bed (decidua and invasive extravillous trophoblast cells) from mid-gestation in the human.

a) IGF-II mRNA and cytokeratin: in situ hybridization was performed for IGF-II mRNA and sections were subsequently immunostained for cytokeratin, to identify extravillous trophoblast cells embedded in the decidua. X560

b) IGF-II mRNA and vimentin: in situ hybridization was performed for IGF-II mRNA and sections were subsequently immunostained for vimentin, to identify the maternal decidual cells. X560

c) IGF-II peptide: immunohistochemistry for IGF-II peptide.

Results from this study demonstrate that IGF-II mRNA is selectively expressed by the invasive extravillous trophoblast cells embedded in the decidual tissue; whereas, IGF-II peptide is distributed in both extravillous trophoblast cells as well as the maternal decidual cells. X560 Courtesy of Dr. V.K.M. Han, Lawson Research Institute, London, ON.


IGF-II mRNA and CYTOKERATIN
IGF-II mRNA and VIMENTIN
IGF-II PEPTIDE

however, IGFBP-1 mRNA is expressed only by the decidual cells (Rutanen et al.,1991), particularly those in the vicinity of the invasive intermediate trophoblast (Han, V.K.M., personal communication; Figure 4). Thus, the biological activity of IGF-II produced by the invasive intermediate trophoblast cells may be influenced in situ by interaction with IGFBP-1 produced by decidual cells in their vicinity.

3.4. Colony Stimulating Factor-1

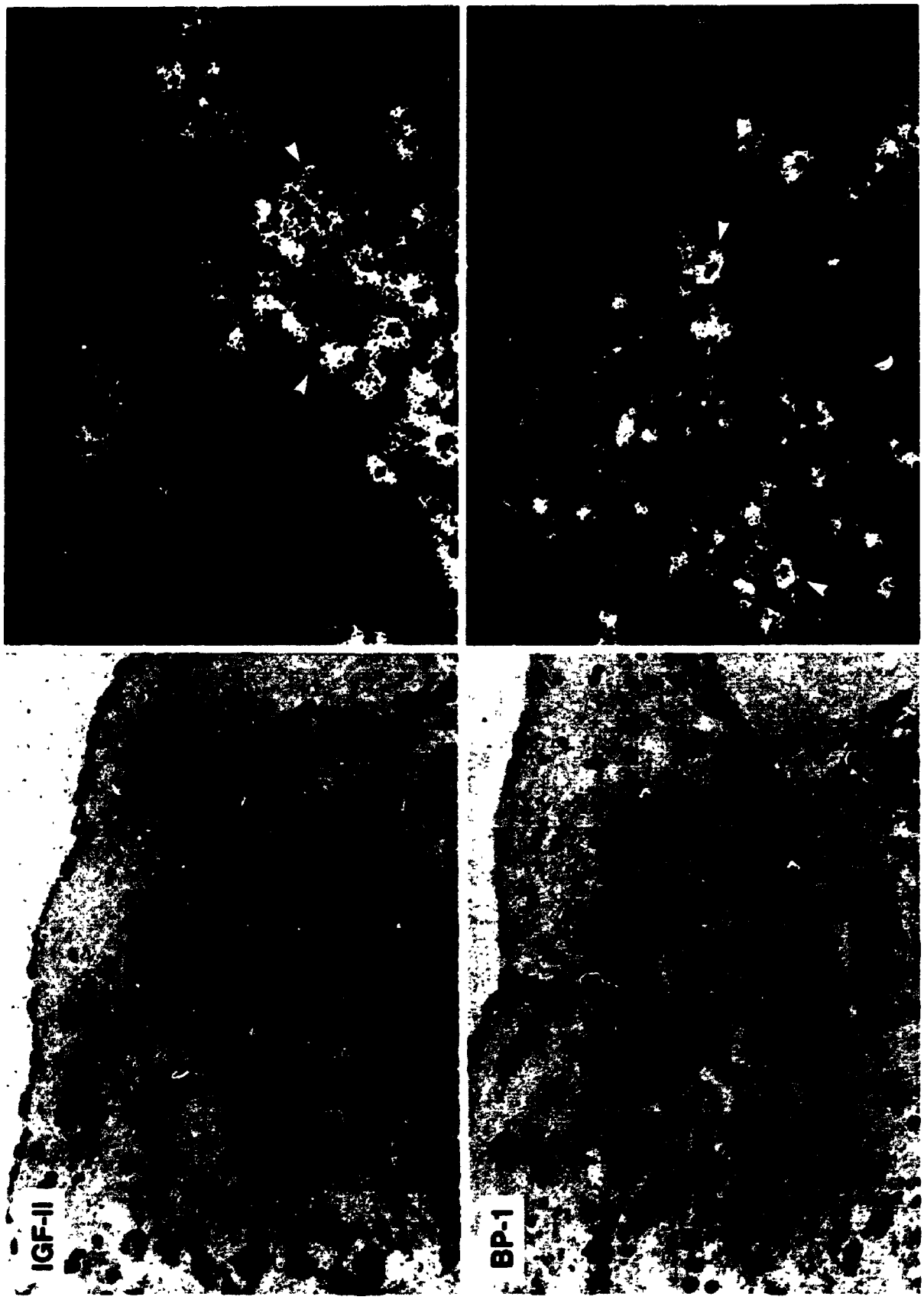
Earlier studies in mice revealed that pregnancy induces a 1000-fold increase in uterine CSF-1 concentration and that this increase is apparently regulated by the female sex hormones oestradiol-17ß and progesterone (Bartocci et al., 1986; Pollard et al., 1987). Recent studies in the human have identified CSF-1 mRNA (by Northern blotting) in the human placenta and the decidua, the expression of which increased during pregnancy (Saji et al., 1990). CSF-1 mRNA was not detected in the nonpregnant endometrium; however, it was detected in the endometrium of a uterus made pseudopregnant by the adminstration of mestranol and norethindrone to a patient prior to total hysterectomy (Saji et al., 1990). The mRNA of the CSF-1 receptor, the *c-fms* protooncogene, has also been detected in the human placenta and the decidua and the expression is found to increase with gestation (Pampfer et al., 1992; Saji et al., 1990). Using the method of RT-PCR, Pampfer et al (1992) have described a trophoblast-specific transcription of exon 1 of *c-fms*. Immunohistochemical analysis of *c-fms* protein in the human placenta and the decidua throughout gestation has revealed its presence during the first trimester on glandular epithelial cells, decidual cells, the syncytiotrophoblast cell

Figure 4: Photomicrographs of sections of the placental bed from mid-gestation in the human.

a) IGF-II: in situ hybridization was performed for IGF-II mRNA and subsequently sections were immunostained for vimentin (b, dark field photomicrograph of the same area as in a). X280

c) BP-1: in situ hybridization was performed for IGFBP-1 mRNA and subsequently sections were immunostained for vimentin (d, dark field photomicrograph of the same area as in c). X280

These results show that decidual cells (vimentin positive cells) in the immediate vicinity of the invasive extravillous trophoblast cells (IGF-II mRNA positive, vimentin negative cells) express high levels of the IGF binding protein (IGFBP)-1 mRNA. Courtesy of Dr. V.K.M. Han, Lawson Research Institute, London, ON.


IGF-II
BP-1

layer and intermediate trophoblast cells; a patchy immunostaining pattern was observed over the cytotrophoblast cells. At later gestational ages the immunostaining was confined to the syncytiotrophoblast cell layer (Pampfer et al., 1992). Thus CSF-1 is present at the human fetomaternal interface and may be involved in both autocrine and paracrine interactions involving trophoblast cell growth and/or differentiation and also decidualization.

4. PLACENTAL GROWTH

4.1. Overview

The growth of the placenta is the net result of the proliferation and differentiation of numerous cell populations. Floating chorionic villi which form early during placental development, as described earlier, are finger-like projections consisting of a layer of cytotrophoblast cells surrounded by a layer of syncytiotrophoblast, and a mesodermal core inclusive of fetal capillaries. As the placenta grows, chorionic villi grow and branch and some will form anchoring villi. The mesodermal core will continue to expand with the growth of the placenta. The expansion of the trophoblast layers result from the proliferation of the cytotrophoblast cells (Richart, 1961; Galton, 1962). The syncytiotrophoblast cell layer is a non-proliferative, terminally differentiated layer of syncytium which arises through the fusion of the underlying cytotrophoblast cells (Boyd and Hamilton, 1970). In anchoring villi, cytotrophoblast cells which sprout from the tips

of villi and invade the maternal decidual tissue are termed extravillous (intermediate) trophoblast cells, which are also capable of proliferation (Boyd and Hamilton, 1970; Clint, 1979; Loke, 1983). Thus, placental growth is dependent upon the proliferation of cytotrophoblast cells. Growth of the villous chorion continues until approximately mid-gestation, thereafter cytotrophoblast proliferation declines and the syncytiotrophoblast becomes the predominant trophoblast cell layer (Loke, 1983). At term the human placenta may be considered a hemodichorial placenta because of the relative lack of villous cytotrophoblast cells (Boyd and Hamilton, 1970).

The studies described above are based on histological observations of the placenta. Recent studies have employed markers for proliferating cells, proliferating cell nuclear antigen (PCNA; King and Blankenship, 1993) and Ki67 (Muhlhauser et al., 1993) on sections of macaque and human placenta, respectively. Results from these experiments showed that syncytiotrophoblast cells were always negative, villous cytotrophoblast cells were positive but their immunostaining decreased near term, and numerous extravillous trophoblast cells of cell columns, cell islands, and the trophoblastic shell were immunoreactive for PCNA or Ki67. These studies provide direct evidence that both villous and extravillous cytotrophoblast cells are highly proliferative cell populations and their proliferation appears to be regulated both spatially as well as temporally (King and Blankenship, 1993; Mulhauser et al., 1993).

4.2. Trophoblast Growth In Vitro

Few laboratories have been able to successfully propagate human trophoblast cells

in vitro (Yagel et al., 1988; Graham et al., 1992; Filla et al., 1993); thus, the information on trophoblast growth promoting and inhibitory molecules is limited.

4.2.1. In Vitro Trophoblast Growth Stimulatory Molecules

Several studies have suggested that the EGF-receptor ligands EGF and TGFα may promote trophoblast growth in situ (Haining et al., 1991; Bissonnette et al., 1992; Lai et al., 1986; Magid et al., 1985; Chen et al., 1988). Most of the above studies were based on immunohistochemical observations of EGF, TGFα or the EGF-receptor or mRNA expression of these factors in the placenta. Concurrent with our studies (described later), direct evidence for a growth stimulatory role of EGF and TGFα was recently demonstrated by Filla et al (1993).

4.2.2. Trophoblast Growth Inhibitory Molecules

Recent studies from this laboratory by Graham et al (1992) have shown that TGFß produced at the fetomaternal interface, by the decidua and to a small extent by trophoblast cells, has an important regulatory role for the growth of first trimester human trophoblasts in vitro. Addition of increasing concentrations of TGFß to cultures of first trimester human trophoblast cells caused a dose-dependent decrease in ^{3}H-TdR incorporation. These investigators also found that addition of anti-TGFß neutralizing antibody to trophoblast cultures caused a minor but significant stimulation of proliferation beyond control levels suggesting an endogenous production of TGFß with antiproliferative activity.

Graham et al (1992) further demonstrated that, in addition to exerting growth

inhibitory effects, TGFß also enhanced the formation of multinucleate cells in these first trimester trophoblast cell cultures. They hypothesized that these multinucleate cells in their cultures were the in vivo equivalent of placental bed giant cells, since the trophoblast cells propagated in their cultures were possibly the in vivo equivalents of intermediate trophoblast cells.

5. PLACENTAL INVASION

5.1. Overview

Trophoblast invasiveness of the uterus is essential for proper implantation of the blastocyst and subsequent development of the placenta in certain species including human. An invasive subpopulation of cytotrophoblast cells migrate and invade the uterine endometrium, including glands and blood vessels (Boyd and Hamilton, 1970; Pijnenborg et al., 1981). Histologically, these processes are marked by the presence of extravillous cytotrophoblast cells in the anchoring villi, cytotrophoblastic shell embedded in the decidua, and dispersed cytotrophoblast cells in the decidua basalis or in the inner myometrial layer of the uterus, as well as endovascular trophoblast cells lining the walls of the uteroplacental (spiral) arteries (Boyd and Hamilton, 1970; Pijnenborg et al., 1981).

In the human, cytotrophoblast cell invasion occurs primarily in the villous chorion and proceeds until about the middle of gestation, thus their invasiveness is regulated in both a spatial and temporal manner (Boyd and Hamilton, 1970). Factors governing these

events have been the focus of research only in recent years (Graham and Lala, 1991, 1992).

5.2. Trophoblast Invasion in Various Mammalian Orders

The classification of placentas from various mammalian orders has largely been based on the degree of trophoblast invasion as seen histologically (Avery, 1965). The "epitheliochorial" type placenta of ungulates is non-invasive, so that trophoblast cells remain in close apposition to the uterine epithelium. Carnivores possess an "endotheliochorial" type of placenta in which trophoblast cells erode the uterine epithelium and underlying connective tissue and come into contact with uterine vessels. In "hemochorial" type placentas found in rodents and anthropoids, trophoblast cells breach the uterine epithelium, invade the uterine stroma, inclusive of glands, and also invade maternal blood vessels, thus becoming bathed in maternal blood. In "hemoendothelial" placentas (of guinea pigs and rabbits) trophoblast cells disappear in places, leaving the fetal chorionic vessels in close proximity with maternal blood.

It is an interesting observation that invasive type of placentation is always accompanied by a decidual cell reaction, whereas, in non-invasive placentation a decidual cell reaction is not necessarily present (Finn and Porter, 1975).

5.3. Sequential Stages of Trophoblast Invasion

Invasiveness is not an exclusive property of trophoblast cells. The ability of cells

to invade basement membranes and migrate through the ECM is required for morphogenesis during normal embryonic development, as well as tissue remodelling in adult life, eg during ovulation, and angiogenesis in the uterus during pregnancy and menstruation. Certain migratory cells in the blood, eg lymphocytes and macrophages also have invasive ability. Finally, invasion is an essential feature during the process of tumor progression and metastasis of cancer cells.

The invasion of a basement membrane by an invasive cell is a multistep process (Liotta, 1986). Cells must initially recognize some component of the basement membrane such as type IV collagen, laminin, fibronectin, vitronectin, heparan sulfate proteoglycan, entactin, or nidogen and attach to it via integrin receptors or other specific receptors. This interaction between the receptors and their ligands may then trigger the expression of genes necessary for the subsequent steps of basement membrane invasion. These subsequent steps include cell detachment, degradation of the basement membrane components, and cell migration through the degraded matrix.

Studies of cellular attachment to basement membrane and ECM components have rapidly advanced during recent years because of the identification of a number of cell surface receptors for the ECM components named as integrins and the development of monoclonal or polyclonal antibodies to various integrin subunits (Ruoslahti et al., 1994), an area beyond the scope of review in this thesis. Likewise, much is known about the process of basement membrane degradation (Mignatti et al., 1986; Liotta et al., 1986); however, there is limited knowledge of the process of cell detachment and migration. The following account is limited primarily to studies of trophoblast invasion.

5.3.1. Attachment to Basement Membrane and/or ECM Components

Attachment of trophoblast cells to the basement membrane substrate may be mediated by laminin binding since it was demonstrated that trophoblast cells adhere better to laminin-coated substrates than to plastic surfaces (Loke et al., 1989). Yagel et al (1988) have also shown that trophoblast cells produce laminin which may aid in attachment.

Recently, Damsky et al (1992) have described the in vivo pattern of integrin expression on cytotrophoblast cells and the presence of extracellular matrix ligands in the first trimester human placenta. Using immunocytochemical methods they found that villous cytotrophoblast cells anchored to the basement membrane were immunoreactive for $\alpha 6$ and ß4 integrin subunits and the ECM components merosin and A-chain-containing laminin. Extravillous cytotrophoblast in the anchoring villus (intermediate trophoblast) stained primarily for $\alpha 5$ and ß1 integrin subunits and had a fibronectin-rich matrix. Extravillous cytotrophoblast cells embedded in the endometrium were immunoreactive for $\alpha 1$, $\alpha 5$, and ß1 integrins but were negative for most ECM antigens. Tenascin immunostaining was found in the stroma of the villus at sites of transition from the floating to the anchoring villus. Based on these findings, Damsky and co-workers (1992) suggested that regulation of integrin expression contributes to the invasive phenotype displayed by extravillous cytotrophoblast cells. It appeared that attachment of trophoblast cells to fibronectin may be an important step for an expression of their invasive phenotype. This hypothesis is consistent with the studies of Feinberg et al (1991) showing the localization of oncofetal fibronectin in the ECM connecting extravillous trophoblasts and trophoblast cell columns to the decidual tissue. Recent

studies from our laboratory have shown that trophoblast cells migrating out of primary cultures of first trimester chorionic villus explants express a similar integrin profile as described for the intermediate trophoblast in vivo (Irving et al., 1993). Questions remained however, whether fibronectin is produced by the migrant trophoblast cells and whether binding to fibronectin is a prerequisite for trophoblast migration.

5.3.2. Detachment from Basement Membrane and Migration

Following their attachment to the basement membrane, invasive cells must be able to detach and migrate through the substrate. Yagel et al have demonstrated that the presence of sialylated ß1-6 branched complex-type ASn-linked oligosaccharides is necessary for the invasiveness of metastatic tumor cells (Yagel et al., 1989) as well as first trimester human trophoblast cells (Yagel et al., 1990). Incubating tumor cells or trophoblast cells in the presence of swainsonine, which inhibits the complex branching of terminal sugars, blocked the ability of both cell classes to detach from the basement membrane, and as a consequence, their ability to invade the basement membrane substrate (Yagel et al., 1989, 1990). Thus, sialylated ß1-6 branched complex-type ASn-linked oligosaccharides may be required for the motility of invasive cells.

5.3.3. Degradation of Basement Membrane Components

Degradation of basement membrane components is an important step in cellular invasion. Invasive cells must be able to produce and activate enzymes capable of breaking down basement membrane components. As stated earlier, basement membrane contains numerous components such as collagen type IV, laminin, fibronectin, entactin,

and heparan sulfate proteoglycan (Liotta et al., 1986). Type IV collagen is the most abundant basement membrane component and therefore, enzymes capable of degrading this molecule are most essential for cellular invasion. Basement membrane degradative enzymes include metalloproteases, serine proteases, cysteine proteases, aspartic proteases, and endoglycosidases. The activity of these degradative enzymes may be neutralized by a variety of naturally occurring inhibitors, which may be nonspecific eg α_2-macroglobulin or specific eg tissue inhibitor of metalloprotease (TIMP's), serine protease inhibitors (serpins), and cysteine protease inhibitors. Thus, degradation of basement membrane is dependent on the balance between the production of matrix degradative enzymes and their natural inhibitors (Liotta, 1989). Their precise role in basement membrane degradation by the trophoblast is reviewed later.

5.3.3.1. Invasion Associated Enzymes

a) Metalloproteases

Matrix metalloproteases (MMP's) are a family of enzymes that are initially synthesized as latent proenzymes which require activation. They are dependent on Zn^{2+} ions for activity, require Ca^{2+} for stability and perhaps activity, and are active at neutral pH (Brown et al., 1990; reviewed by Stetler-Stevenson, 1990; reviewed by Werb, 1989; reviewed by Khokha and Denhardt, 1989). Certain important members of the MMP family are: (1) 72 kDa type IV collagenase (gelatinase A; MMP-2), (2) 92 kDa type IV collagenase (gelatinase B; MMP-9), (3) interstitial collagenase (MMP-1), (4) stromelysin (MMP-3), (5) stromelysin-2 (MMP-10), and (6) PUMP-1 (matrilysin; MMP-7). Activation of these enzymes requires autoproteolytic removal of 80 amino acids from the

amino terminal (Brown et al., 1990; reviewed by Stetler-Stevenson, 1990). All enzymes may be activated in vitro by treatment with organomercurial compounds, chaotropic agents or certain proteases such as plasmin. Activation of these metalloproteases is a sequential process starting with the latent proenzyme to the active proenzyme to the active enzyme (reviewed by Stetler-Stevenson, 1990). In vivo activation of the metalloproteases is less well understood. It is suggested that plasmin may be involved in their in vivo activation (He et al., 1989; Mignatti et al., 1986; Yagel et al., 1988). Another important feature of the metalloproteases is that all are inhibited by TIMPs (reviewed by Stetler-Stevenson, 1990; reviewed by Werb, 1989).

(1) 72 kDa type IV collagenase

The 72 kDa type IV collagenase has also been termed 68 kDa gelatinase, gelatinase A, as well as matrix metalloprotease (MMP)-2. It has been cloned and characterized from H-ras transformed human bronchial epithelial cells (Collier et al., 1988). It is produced by many normal human cell types such as fibroblasts (Salo et al., 1985), macrophages (Garbisa et al, 1986), endothelial cells (Kalebic et al., 1983) and trophoblast cells (Graham and Lala, 1990) as well as many human tumor cell types such as colonic and gastric adenocarcinomas (Garbisa et al., 1990), melanomas (Hendrix et al., 1990), and mammary adenocarcinomas (Monteagudo et al., 1990). A direct correlation between tumor cell invasiveness and 72 kDa type IV collagenase production has been noted in many human tumors (reviewed by Stetler-Stevenson, 1990). The 72 kDa type IV collagenase enzyme is not only capable of degrading type IV collagen but also has significant proteolytic activity against denatured collagens, type V collagen, type

VII collagen, and fibronectin but not against type I collagen or laminin (Collier et al., 1988; Werb, 1989).

(2) 92 kDa type IV collagenase

Like the 72 kDa type IV collagenase the 92 kDa type IV collagenase also has different names including gelatinase B and MMP-9. It has been cloned and sequenced from SV40 transformed human lung fibroblasts (Wilhelm et al., 1989). It is present in many normal human cell types including polymorphonuclear leukocytes (Hibbs et al., 1985; Murphy et al., 1982), macrophages (Mainardi et al., 1980, 1984), and cytotrophoblast cells (Fisher et al., 1989; Librach et al., 1991), as well as some human tumor cell lines. The 92 kDa type IV collagenase enzyme is capable of degrading type V and type IV collagen, but does not degrade type I collagen, proteoglycan, laminin, or fibronectin.

Both the 72 kDa and 92 kDa type IV collagenases have three domains. The carboxyl-terminal domain and the amino-terminal domain are homologous to those of the other metalloproteases; however, both also have a third domain which is homologous with the collagen-binding domain of fibronectin (Collier et al., 1988; reviewed by Stetler-Stevenson, 1990). The ability of both enzymes to bind to and degrade gelatin is due to this third domain, and thus they are also described as gelatinases.

(3) Interstitial Collagenase

Interstitial collagenase is a group of enzymes also known as MMP-1 capable of degrading collagens types I, II, III, VIII, and X, but does not cleave basement membrane

collagen type IV or type V collagen (reviewed by Goldberg et al., 1986; reviewed by Werb, 1989). Interstitial collagenase is produced by many cells such as, macrophages, fibroblasts, synovial cells, osteoblasts, chondrocytes, and endothelial cells (reviewed by Werb, 1989).

(4) Stromelysin

Stromelysin is another major metalloprotease also known as proteoglycanase, MMP-3, transin, and neutral proteinase. It is produced in cultures of fibroblasts, synovium, and certain other cells (reviewed by Werb, 1989). Stromelysin degrades proteoglycans, collagen types IV, V, and VII, denatured type I collagen, laminin, fibonectin, elastin, α_1-proteinase inhibitor, and immunoglobulins (reviewed by Werb, 1989). Murphy et al (1989) have suggested that stromelysin is as effective as or even more effective than the type IV collagenase in degrading type IV collagen, and thus may be important for basement membrane degradation. Stromelysin has also been implicated in tumor metastasis (reviewed by McDonnell and Matrisian, 1990).

(5) Stromelysin-2

Stromelysin-2 is also known as MMP-10 and transin-2. Stromelysin-2 degrades fibronectin, denatured collagens, and native types III, IV, and V collagen. Like stromelysin, stromelysin-2 has also been implicated in tumor progression and metastasis (reviewed by McDonnel and Matrisian, 1990).

(6) Pump-1

Pump-1 (recently renamed as matrilysin) is a third stromelysin-like enzyme that was identified by screening a cDNA library prepared from human tumor samples (Mueller et al., 1988). The cDNA identified had an amino acid sequence that suggested the protein product was a putative metalloprotease, hence the name pump-1 (Mueller et al., 1988). Pump-1 has been given the MMP designation of MMP-7. Pump-1 is most likely identical to the small metalloprotease isolated from the rat uterus called small metalloprotease of the uterus (reviewed by McDonnell and Matrisian, 1990), which appears to be involved in uterine involution following pregnancy (Quantin et al., 1989; Woessner and Taplin, 1988). It is produced by glandular epithelial cells of the human uterus (Matrisian, personal communication). Like stromelysin and stromelysin-2, pump-1 degrades fibronectin and denatured collagen, however, it does not degrade intact collagens (Quantin et al., 1989; Woessner et al., 1988). Pump-1 has also been implicated in tumor progression; it was detected in 8 of 10 samples of malignant stomach cancer (reviewed by McDonnell and Matrisian, 1990).

b) Serine Proteases

Serine proteases are characterized by a serine residue at their catalytic site. They are the largest family of mammalian proteases and their inhibitors represent 10 percent of all plasma protein (reviewed by Werb 1989). Many of the enzymes involved in the cascades of coagulation, fibrinolysis, and complement activation are serine proteases (reviewed by Werb, 1989). Serine proteases include thrombin, plasmin, plasminogen activators, trypsin, chymotrypsin, elastase, cathepsin G, and plasma kallikrein (reviewed

by Werb, 1989). Plasmin and plasminogen activators are required for invasiveness of many cells, including trophoblasts and thus will be discussed further.

(1) Plasmin

Plasmin is derived from its inactive precursor, plasminogen, after activation by urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) (Collen, 1987). It acts by cleaving the carboxyl side of peptide bonds containing lysyl residues (Collen, 1987). Plasmin is capable of degrading fibrin as well as cartilage proteoglycan and although it has no direct effect on collagen it can activate procollagenase (reviewed by Werb, 1989). Jensen et al (1989) has demonstrated the presence of plasmin on the surface of human trophoblast cells.

(2) Plasminogen Activators

There exists two distinct genes for plasminogen activators, they are tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) both of which contribute to the conversion of plasminogen to plasmin (Collen, 1987). uPA is initially secreted as a single chain proenzyme of 55 kDa which must be cleaved for activation into two chains of 20 kDa and 34 kDa that are connected by a disulfide bridge (Collen, 1987). This activation of uPA may be performed by plasmin itself (Collen, 1987). tPA is also secreted in a latent form of 72 kDa which requires removal of a peptide for its activation. Plasmin may also convert tPA into a two-chain form however, both forms of tPA are active (Collen, 1987). Plasminogen activators are secreted by macrophages, fibroblasts, synovial cells, endothelial cells, polymorphonuclear leukocytes

(reviewed by Werb, 1989), and human trophoblast cells (Yagel et al., 1990). Both the active and the proenzyme form of uPA may bind to a specific saturable receptor present on macrophages (Collen, 1987; Blasi et al., 1987), fibroblast (Collen, 1987; Vassalli et al., 1985) and human cytotrophoblast cells (Zini et al., 1992) via its non-catalytic site, so that the active sites of uPA on the cell membrane may provide a polarized invasion front.

c) Cysteine Proteases

Cysteine proteases comprise the lysosomal enzymes cathepsin B and cathepsin L. The catalytic sites of cysteine proteases require cysteine and histidine residues (Barrett and Kirschke, 1981). Both are synthesized as higher molecular weight forms and are activated in lysosomes to enzymes of molecular weight 25 kDa; however, cathepsin L may be secreted in both forms by inflammatory macrophages and tumor cells (reviewed by Werb, 1989). These enzymes are active at acid pH and degrade denatured collagen, type I collagen, and heparan sulphate proteoglycan (reviewed by Werb, 1989).

d) Aspartic Proteases

Aspartic proteases are characterized by containing aspartic acid residues integrated in their catalytic mechanisms. Cathepsin D is the most prominent protease in this family found in lysosomes and acting at acid pH (reviewed by Werb, 1989). Cathepsin D is present in lysosomes of fibroblasts and macrophages (Erickson et al., 1981). Substrates for cathepsin D include cartilage proteoglycans; however, an acidic pH is essential for the extracellular activity of cathepsin D (reviewed by Werb, 1989).

Cathepsin D has been implicated in tumor progression and is correlated with a poor prognosis in breast tumors (reviewed by Rochefort et al., 1990).

e) Endoglycosidases

Endoglycosidases are a family of enzymes involved in the degradation of glycosaminoglycans (Liotta, 1986). Glycosaminoglycans are integral components of proteoglycans. They include molecules such as hyaluronic acid, chondroitin sulfate, keratan sulfate, dermatan sulfate, heparan sulfate, and heparin. They constitute an important part of the extracellular matrix and may be important in the structural organization of the basement membrane which contains heparan sulphate proteoglycan (reviewed by Liotta, 1986).

5.3.3.2. Natural Inhibitors of Proteases

a) α_2-Macroglobulin

α_2-macroglobulin (α_2-M) is a non-specific protease inhibitor, being able to inhibit metalloproteases, serine proteases, cysteine proteases, and aspartic proteases (reviewed by Werb, 1989). It is produced essentially by the liver but may be produced locally by macrophages. α_2-M has a molecular weight of 718 kDa and is comprised of four identical subunits, each subunit possessing a short amino acid sequence which is highly sensitive to proteases. Proteolytic cleavage of one of these sites results in a conformational change in α_2-M and physically traps the protease and forms covalent bonds with the protease; thus, α_2-M inhibits only active proteases. The binding of proteases to α_2-M is in a molecular ratio of 2:1. α_2-M-protease complexes are rapidly

removed by binding to α_2-M receptors present on macrophages followed by endocytosis (reviewed by Werb, 1989).

b) Metalloprotease Inhibitors

α_2-M is the major inhibitor of metalloproteases present in plasma and synovial fluid of joints afflicted with rheumatoid arthritis. However, there exists a family of tissue inhibitors of metalloproteases (TIMPs) responsible for controlling tissue levels of the metalloproteases.

(1) TIMP-1

TIMP-1 is a glycoprotein with a molecular weight of 28.5 kDa (Welgus and Stricklin, 1983; Welgus et al., 1985). The gene encoding human TIMP-1 has been cloned, sequenced, and mapped to a region on the X-chromosome (Carmichael et al., 1986; Docherty et al., 1985; Mahtani and Willard, 1988). TIMP-1 binds to activated metalloproteases at an equimolecular ratio, to inhibit metalloprotease activity (reviewed by Stetler-Stevenson, 1990). Most cells capable of producing metalloproteases are also capable of producing TIMP-1; thus, the net collagenolytic activity for these cells is the result of the balance between activated metalloproteases and TIMP-1 levels (reviewed by Stetler-Stevenson, 1990). TIMP-1 has been inversely linked to the tumorigenic ability of certain cells. Insertion of antisense TIMP-1 mRNA has been reported to induce tumorigenic and metastatic ability in mouse 3T3 cells (Khokha et al., 1989), whereas upregulation of TIMP-1 mRNA in tumor cells has been shown to reduce invasion (Khokha et al., 1992a) and tumorigenic ability (Khokha et al., 1992b) in B16F10

melanoma cells.

(2) TIMP-2

TIMP-2 is a nonglycosylated 21 kDa secreted protein of 194 amino acids, which has been cloned, sequenced and found to have 65% homology with TIMP-1 at the amino acid level (Stetler-Stevenson et al., 1989, 1990). Unlike TIMP-1, TIMP-2 preferentially binds to the latent proenzyme as well as the activated form of the 72 kDa type IV collagenase enzyme at a 1:1 molar ratio; however, its binding to these two forms of the enzyme appears to be at different sites (reviewed by Stetler-Stevenson, 1990). Binding of TIMP-2 to the latent proenzyme form of the 72 kDa type IV collagenase does not result in total inactivation of the 72 kDa type IV collagenase. This enzyme may still become activated but its type IV collagenolytic activity is decreased 20 fold (Fridman et al., 1993).

(3) TIMP-3

TIMP-3 has recently been cloned and sequenced from a mouse cDNA library. It has 80% amino acid homology with chicken inhibitor of metalloprotease (Chimp)-3. Chimp-3 is found exclusively in the ECM and disrupts the adherence of chicken embryo fibroblasts to the ECM. Chimp-3 also promotes the growth of non-transformed cells and the expression of a transformed phenotype (reviewed by Denhardt et al., 1993).

c) Serine Protease Inhibitors

Specific inhibitors of serine proteases are members of a multigene superfamily

known as serpins (Carrell and Boswell, 1986). Serpins are glycoproteins ranging in molecular weights from 50 to 100 kDa and include the plasma inhibitors eg α_2-proteinase inhibitor, antithrombin III, C1-inhibitor, α_2-antiplasmin, α_1-antichymotrypsin, and the tissue inhibitors eg protease nexins-1, 2, and 3, and the plasminogen activator inhibitors (PAI)-1 and 2. PAI-1 and PAI-2 inhibit both uPA and tPA (reviewed by Werb, 1989).

d) Cysteine Protease Inhibitors

Extracellular inhibitors of cysteine proteases include a specific inhibitor called α-cysteine proteinase inhibitor (αCPI) as well as the nonspecific inhibitor α_2-M (Travis and Salvessen, 1983; Barrett et al., 1986). αCPI exists in a variety of forms with molecular weights $\geq$ 60 kDa. Two cytoplasmic inhibitors also exist, known as cystatins each having a molecular weight of 13 kDa (Barrett et al., 1986).

5.4. Mechanisms of Trophoblast Invasion

5.4.1. In Vivo Studies on Trophoblast Invasion

During implantation in the human, the outer cells of the blastocyst (trophoblast cells) breach the uterine epithelium, degrade the underlying basement membrane and penetrate the endometrium. As the placenta develops, trophoblast cells invade the endometrial stroma, glands, and blood vessels. This process of trophoblast invasion is histologically evident until approximately week 17 of human gestation (Boyd and Hamilton, 1987).

In the early to mid sixties Kirby (1960, 1963a, 1963b, 1965) designed experiments to examine the invasive ability of mouse trophoblast transplanted orthotopically into the uterus or ectopically in the testes, or underneath the kidney capsule. Trophoblast invasion, as seen histologically, was greater in the non-pregnant uterus and extra-uterine sites than in the pseudopregnant or the pregnant uterus. Results from these experiments suggested that mouse trophoblast cells were inherently invasive and the maternal decidual tissue may control the extent of trophoblast invasion.

Combining histological, histochemical, and biochemical methods Denker (1977) showed that, in the rabbit and the cat, trophoblast attachment, penetration, and degradation involved proteases and glycosidases. The proteases implicated in this process were endopeptidases, thiol proteinases, and serine proteases.

In the human a number of pregnancy associated diseases are associated with abnormal trophoblast invasion, for example; ectopic pregnancy and placenta accreta show high degrees of trophoblast invasion and a poor decidual response (Billington, 1971), while preeclampsia exhibits shallow invasion, in particular, a poor invasion of the spiral arteries.

5.4.2. In Vitro Studies on Trophoblast Invasion

5.4.2.1. In Vitro Invasion Assays

Invasion is a property shared by certain normal cells, in particular embryonic cells, and malignant tumor cells. Tumor biologists have employed a variety of in vitro invasion assays using natural tissue substrates eg chick chorio-allantoic membrane (Hart

and Fidler, 1978; Chambers et al., 1982), chick embryonic heart fragments (Mareel et al., 1979), bovine lens capsule (Starkey et al., 1984), and epithelium free human amnion (Liotta et al., 1980; Mignatti et al., 1986; Yagel et al., 1988) or reconstituted gels of basement membrane components such as matrigel (Repesh et al., 1989) to measure invasiveness of tumor cells. Our laboratory initially employed an amnion invasion assay (Yagel et al., 1988; Graham and Lala, 1991) and later a matrigel invasion assay (Graham and Lala, 1992; Graham et al., 1993a) to measure the invasive ability of first trimester trophoblast cells in culture. In both assays, trophoblast cells are prelabeled with ^{125}I-deoxyuridine (dUR) or ^{3}H-TdR and placed on the invasion substrate. In the amnion invasion assay, the invasion at a particular time point is then measured as the percent of radioactivity retained in the body of the amniotic membrane resulting from the radio-labeled cells in transit; in the matrigel invasion assay, this is measured as the percent of radioactivity accumulating in a lower well because of transgression of the matrigel barrier by the radioactive cells.

5.4.2.2. Matrix Degradation and Invasion by Human Cytotrophoblast Cells

To assess the matrix degrading properties of human trophoblast cells, Fisher et al (1985) plated chorionic villi from first, second, and third trimesters on ^{3}H-leucine-labeled ECM isolated from two cell lines. The extracellular matrix produced by the cell lines was found to contain basement membrane-specific molecules, including type IV collagen, laminin, heparan sulfate proteoglycan, and entactin, thus representing a suitable basement membrane-like substrate. Attachment and matrix degradation were then assessed following the plating of chorionic villi. Results from these experiments revealed

that chorionic villi from first and second trimesters adhered to the matrix equally well, however, villi from third trimester did not adhere. Electron microscopic analysis of the adherent cells showed that they had the morphology of cytotrophoblasts. Assay of the conditioned media for ^{3}H-label released from the matrix indicated that only cytotrophoblast cells from first trimester chorionic villi were capable of degrading the basement membrane-like matrix. Subsequently, Fisher et al (1989) isolated human cytotrophoblast cells from first, second, and third trimester by enzyme dispersion and density gradient centrifugation and used them in a matrigel invasion assay to confirm that only first trimester cells were capable of matrix degradation. They further reported that 1,10-phenanthroline, an inhibitor of metalloproteases and not other proteases inhibitors blocked the degradative activity of the trophoblast cells, indicating that the metalloproteases were essential for invasion. Fisher et al (1989) concluded that trophoblast invasiveness was developmentally regulated during the course of human gestation.

In a follow up study, Librach et al (1991) from the same laboratory, examined the role of specific enzymes on trophoblast invasion of matrigel. Incubation of cytotrophoblast cells with an affinity purified polyclonal anti-92 kDa type IV collagenase antibody during the invasion assay, under serum-free conditions, blocked the invasiveness of these cells suggesting that the 92 kDa type IV collagenase plays a critical role. Since the plasmin inhibitors, aprotinin and α_2-antiplasmin, only partially blocked this invasion, they concluded that trophoblast invasion was plasmin-independent. However, the results may also be interpreted differently. This inhibition may have been partial due to the inactivation of only cell membrane associated plasmin since no plasmin was present in

their incubation media (Graham and Lala, 1992). Plasmin has been reported on the cell membrane of trophoblast cells (Jensen et al., 1989) and it has also been demonstrated that trophoblast cells possess high numbers of high affinity uPA receptors (Zini et al., 1992), which remain saturated with endogenously produced single chain form of uPA.

In contrast to the report by the Fisher group, findings by Kliman and Feinberg (1990) demonstrated that both first trimester and term human cytotrophoblast cells were capable of degrading matrigel. Recent studies by Graham and Lala (1992) have shown that term trophoblast cells isolated according to the procedure described by Kliman et al (1986) were invasive in an in vitro amnion invasion assay. These findings would suggest that trophoblast invasion is controlled by the microenvironment, rather than in a pre-programmed manner.

In order to investigate the mechanisms of trophoblast invasiveness, Yagel et al (1988), in this laboratory, developed a technique to grow long term cultures of first trimester human trophoblast cells, which has subsequently been modified by Graham et al (1992). Yagel et al (1989) found that first trimester trophoblast cells were as invasive as metastatic tumor cells when tested with in vitro amnion invasion assays. Invasion assays conducted in the presence of metalloprotease inhibitors (1,10-phenanthroline and recombinant TIMP-1), serine protease inhibitors (trasylol and EACA), anti-plasminogen and anti-uPA antibodies, and a metalloprotease activator (mersalyl) revealed that inhibition of both proteases blocked invasion and mersalyl enhanced invasion even in the presence of the serine protease inhibitor trasylol. Lala et al (1989) also observed that collagenase activity in trophoblast cultures was reduced in serum-free (plasminogen deficient) media and when incubated in the presence of trasylol, a serine protease

inhibitor. Taken together, these results suggest that metalloproteases are the final mediators of first trimester human trophoblast cell invasiveness and that plasmin, a serine protease, is required for metalloprotease activation. As stated earlier, plasmin is derived from plasminogen after activation by uPA or tPA (Collen, 1987). Indeed, first trimester (Yagel et al., 1990) and term (Queenan et al., 1987) cytotrophoblast cells have been shown to produce uPA; they also express saturable uPA binding sites (Zini et al., 1992).

Controversy exists as to which metalloprotease is the principal mediator of trophoblast invasion. Recent work from this laboratory by Graham and Lala (1991) using Northern blot analysis demonstrated that the 72 kDa type IV collagenase is the major metalloprotease expressed by first trimester invasive human trophoblast cells in culture. This is in contrast to the work of Librach et al (1991) who found that antibodies against the 92 kDa type IV collagenase blocked first trimester trophoblast invasion. This discrepancy may possibly be explained by the use of different subpopulations of first trimester cytotrophoblast cells. Immunostaining has demonstrated an abundance of the 72 kDa type IV collagenase enzyme in first trimester human placenta (Fernandez et al., 1992). Possibly both type IV collagenase species are important for trophoblast invasion, since anti-sense oligonucleotides directed against certain domains of both collagenase species have been found to block trophoblast invasion (Lala and Connelly, 1994).

In summary, first trimester human cytotrophoblast cells are highly invasive cells utilizing metalloproteases as well as serine proteases for their matrix degrading abilities.

5.5. Control of Trophoblast Invasion in situ

Since human first trimester cytotrophoblast cells are endowed with a high invasive ability, Graham and Lala (1991, 1992) asked the question what protects the uterus from destruction by the invasive trophoblast? Provided with clues from early experiments by Kirby (1960, 1963a, 1963b, 1965) that the maternal decidua may play a protective role, Graham and Lala tested whether the maternal decidua produces soluble factor(s) which influence trophoblast invasion in vitro. Graham and Lala (1991; 1992) found that decidual cell conditioned media blocked trophoblast invasion, and that this anti-invasive effect could be abrogated by the addition of an anti-TGFß neutralizing antibody or an anti-TIMP-1 antibody, and was mimicked by exogenous TGFß. These findings revealed that both TGFß as well as TIMP-1 were candidate anti-invasive molecules. Results of trophoblast invasion assays performed in the presence of anti-TGFß or anti-TIMP-1 antibody alone revealed a slight stimulation in invasion over control levels suggesting that trophoblast cells themselves produce a small amount of TGFß and TIMP-1 (Graham and Lala, 1991, 1992).

In order to investigate the mechanisms of the anti-invasive effect of TGFß, Graham and Lala (1991, 1992) incubated first trimester trophoblast cells in the presence of exogenous TGFß or anti-TGFß antibody and then examined the conditioned media for type IV collagenous activity and examined the mRNA expression of TIMP-1, TIMP-2, 72 kDa type IV collagenase, and interstitial collagenase in trophoblast cells by Northern analysis. They found that the addition of TGFß reduced type IV collagenase activity and that this was due to a TGFß upregulation of TIMP-1 mRNA and protein. Thus, decidua-

derived, and to a minor extent trophoblast-derived TGFß decreases first trimester human trophoblast invasion by upregulating TIMP-1 production by trophoblast cells and therefore shifting the balance of collagenase and inhibitor in favour of the inhibitor. Graham and Lala (1991) further showed that decidua-derived TGFß was secreted in an inactive form, whereas trophoblast-derived TGFß was active. They suggested that decidua-derived TGFß is possibly activated by trophoblast-derived proteases eg plasmin.

Recently, Graham et al (1993a) have shown that the anti-invasive mechanisms of TGFß may be two-fold: (1) stimulation of TIMP-1 production; and (2) decrease of uPA production. Zymographic analysis revealed that TGFß reduced the levels of uPA in trophoblast cultures. A reduction of uPA would reduce the amount of plasmin and thus reduce the levels of type IV collagenase, since collagenase activation in trophoblast cells is plasmin-dependent.

The question remains, however, what regulates TGFß production by decidual and trophoblast cells and are there molecules which effect the activation state of TGFß in situ?

6. PROPAGATION AND CHARACTERIZATION OF PURE FIRST TRIMESTER INVASIVE HUMAN TROPHOBLAST CELL CULTURES

An understanding of the roles of specific growth factors on placental growth and invasion had largely been hindered by methodological limitations in propagating pure human trophoblast cells in culture. These limitations have recently been overcome in our laboratory by the development of an explant culture method (Yagel et al.,1989) which

has been further refined (Graham et al.,1992). This method involves the culturing of mechanically derived fragments of first trimester chorionic villi, which allows proliferative and invasive trophoblast cells to migrate out of the these explants. The migrant adherent trophoblast cells are subsequently passaged. Approximately 30% of these villus explants results in migrant cells containing 100% pure trophoblast cells (as identified by cytokeratin immunostaining); whereas, the remaining explants give rise to migrant cells representing a mixture of fibroblasts and trophoblasts (as identified by cytokeratin and vimentin immunostaining respectively) (Irving et al.,1993) with either cell type predominating. Pure normal trophoblast cell lines have been derived from a passage of cells from the 100% cytokeratin positive cultures. Early passage trophoblast cells used for functional studies were also extensively phenotyped for a number of other markers. One hundred percent of these cells expressed placental alkaline phosphatase, urokinase type plasminogen activator (uPA) (Graham et al.,1992), high affinity uPA receptors (Zini et al.,1992) and HLA antigen framework region (as recognized by W6/32 antibody) (Graham et al.,1993b). These cells were negative for 63D3 antigen (a macrophage marker) and factor VIII (an endothelial cell marker) (Graham et al.,1992). During early passages hCG production declines rapidly (Graham et al.,1992). Finally, these cells are immunoreactive for IGF-II peptide and express IGF-II mRNA as demonstrated by Northern analysis and in situ hybridization (Graham et al.,1993b). Human intermediate trophoblast cells in situ also express high levels of IGF-II mRNA throughout gestation (Han, V.K.M., personal communication). Taken together, the phenotype of the trophoblast cells grown in culture indicates that they belong to the invasive intermediate trophoblast subpopulation.

The trophoblast cells propagated in vitro senesce after 12-14 passages. However, our laboratory has recently succeeded in extending their life span after transfection with the SV40 large T antigen (Graham et al.,1993a).

III. HYPOTHESIS

Factors produced locally at the human fetomaternal interface regulate trophoblast growth and invasiveness in an autocrine or paracrine manner.

Studies reported in this thesis were designed to identify some of these factors and elucidate their mechanisms of action. This was achieved by (a) immunolocalization of some of the putative locally-active factors in the human placenta and decidua in situ at various gestational ages, (b) measuring the effects of these factors on the proliferative and invasive ability of first trimester human trophoblast cells propagated in vitro, and (c) measuring the in vitro effects of these factors on the trophoblast expression of important invasion-associated enzymes and their inhibitors.

IV. OBJECTIVES

The objectives of this thesis are:

(1) To further characterize first trimester human trophoblast cells grown in vitro according to the explant culture method adapted by Graham et al (1992).

(2) To immunolocalize growth factors or molecules at the human fetomaternal interface throughout gestation which may have important roles on trophoblast growth or invasion.

(3) To investigate the effect of numerous locally-derived growth factors on the in vitro proliferation of first trimester human trophoblast cells.

(4) To investigate the effect of the same growth factors on trophoblast invasion using an in vitro matrigel invasion assay.

(5) To examine the effects of these growth factors on the levels of mRNA expression of the invasion regulatory molecules such as the 72 and 92 kDa type IV collagenases, TIMP-1 and TIMP-2.

(6) To examine whether certain choriocarcinoma cell lines have an altered respond to the growth factors.

V. MATERIALS AND METHODS

1. CELL LINES USED

Normal first trimester human trophoblast cells propagated in this laboratory as well as commercially available choriocarcinoma cell lines were employed in experiments. Choriocarcinoma cell lines JAR and JEG3 were obtained from the American Type Culture Collection (ATCC, Rock ille, MD). The choriocarcinoma cell line BeWo was a generous gift from Dr. J. Mitchell (Montreal, QE).

2. ESTABLISHMENT OF FIRST TRIMESTER HUMAN TROPHOBLAST CELL CULTURES

Human first trimester trophoblast cell cultures were established according to the procedure described by Graham et al (1992). Briefly, chorionic villi were collected after elective terminations of pregnancy in the first trimester and rinsed in cold RPMI-1640 medium (Grand Island Biological Company, Grand Island, NY). Villi were then mechanically minced with fine scissors, washed in medium, and the villus fragments were cultured as explants in RPMI-1640 containing 10% fetal calf serum, 200 μg/ml streptomycin, 200U/ml penicillin, and 0.50 μg/ml amphotericin (RPMI complete medium). After 2-3 days, nonadherent explants and cells were removed and discarded. Remaining adherent explants were maintained in culture for another 1-2 weeks to allow trophoblast cell growth and migration out of the explants. Cells were passaged and

characterized. Only cultures resulting in 100% pure trophoblast cells after passage, as determined by phenotype, were used in experiments.

Some of the primary explant cultures were used for routine (n=2) and immuno-electron microscopy (n=3). In these cases, the chorionic villus fragments were plated in chamber slides (Tissue-Tek, Nunc, Naperville, IL) coated with type IV collagen (Sigma). Explants remained in culture for one week before processing for electron microscopy.

2.1. Further Characterization of Migrant Cel's from First Trimester Human Chorionic Villus Explants

2.1.1. Electron Microscopic Characterization of Migrant Cells from First Trimester Chorionic Villous Explants

Fixation, dehydration and epoxy embedding was done in situ with the explants and collagen matrix attached to the glass microscope slide of the chamber slide. Culture medium was removed and chamber wells were flooded with 1.6 % glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. Post-fixation was done with 1% osmium tetroxide in distilled water. Osmium was rinsed out with distilled water, the chamber slide was immersed in a dish of water, the chamber wells were lifted off, the slide around the explant culture was scored with a diamond pen and the slide broken to give rectangles containing the explant culture. The glass rectangles of explants were transferred to medicine cups, cell side up, and then dehydrated in ethanol and infiltrated with epoxy

resin. The infiltrated specimens were then placed, cell side down, onto prepolymerised epoxy blocks and then cured overnight at 60 °C. The glass slide was separated from the explant culture by repeatedly freezing and thawing in liquid nitrogen. Thin sections were cut through the area of the matrix immediately adjacent to the glass surface and also at levels near the explant-matrix interface. Sections were counterstained with uranyl acetate and lead citrate.

2.1.2. Immuno-electron Microscopic Characterization of Migrant Cells from First Trimester Chorionic Villus Explants

Fixation, dehydration and epoxy embedding was done in situ. Culture medium was removed and chamber wells were flooded with 1.6 % glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. Aldehyde groups were quenched using 0.2% sodium borohydride in distilled water. Glass rectangles containing the explants (hereafter called 'samples') were made as described above and the samples transferred to medicine cups. The samples were dehydrated in ethanol and infiltrated in Lowicryl K4M (1 hour in 50% Lowicryl in ethanol & 16 hrs in 100% lowicryl). Samples were then placed in resin-filled gelatin capsules and the blocks were polymerized by UV irradiation at 4 °C for 2 days. The resin around the glass rectangles was removed with a blade and the glass removed by allowing a drop of water to flow through the interface. Thin sections were collected on Butvar-coated nickel grids.

Post-embedding immunogold labeling was done using protein A-gold. Grids bearing explant sections were incubated in 1% bovine serum albumin (BSA) in phosphate

buffered saline (PBS) for 10 min. and then incubated for 1 hour in primary antibody. Grids treated with monoclonal murine antibodies (listed below) were then placed in a drop of rabbit anti-mouse IgG for 30 minutes. Unbound antibody was rinsed off in a stream of PBS and the grids were then placed in protein A-gold (20nm) for 30 min. Grids were rinsed in distilled water, dried and then counterstained in uranyl acetate and lead citrate.

Antibodies used:

1. Anti-human cytokeratin, mouse monoclonal, AE1/AE3 (pooled antibodies to basic and acidic cytokeratins), Boehringer Mannheim (Montreal, PQ), 1:100
2. Anti-human fibronectin, rabbit polyclonal, Dako (Carpinteria, CA), 1:200.
3. Anti-human placental lactogen, rabbit polyclonal, Dako, 1:100.
4. Anti-human oncofetal fibronectin, mouse monoclonal; supernatant from hybridoma FDC-6, ATCC.

2.1.3. Characterization of First Trimester Trophoblast Cells Propagated in Culture

Trophoblast cell cultures established by the earlier described technique were previously characterized for a number of phenotypic markers (Graham et al., 1992). Trophoblast cells propagated in vitro expressed cytokeratins 8 and 18, placental alkaline phosphatase, uPA, uPA receptors, produced variable amounts of hCG, and were negative for 63D3, a macrophage marker, and Factor VIII, an endothelial cell marker (Graham et al., 1992). Cells from early passage (2-4), as reported by Graham et al (1992) were

characterized further. They were immunostained for NDOG5, human placental lactogen (hPL), and proliferating cell nuclear antigen (PCNA) as follows. Trophoblast cells were expanded in culture and subsequently plated into chamber slides (Tissue-Tek, Nunc, Naperville, IL) at a concentration of 10^4 cells/chamber and allowed to adhere overnight. The following day the media was removed, cells were rinsed with PBS, fixed with ice-cold methanol at -20°C for 15 minutes, and washed with PBS. Chambers were flooded with 10% normal horse serum (for NDOG5 and PCNA immunostaining) or 10% normal goat serum (for hPL immunostaining) for 1 hour at room temperature followed by a PBS wash. The primary Abs NDOG5 (1:5 dilution; a kind gift from Dr. S. Shorter, Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford, UK) and PCNA (1:1700; Biogenex, San Ramon, CA), both mouse monoclonal Abs, and hPL (1:200), a rabbit polyclonal Ab (Dako), were then applied and incubated overnight at 4°C. The next day, slides were washed in PBS and incubated with either a biotinylated horse anti-mouse Ab (7.5 μg/ml; Vector) for cells incubated with NDOG5 and PCNA Abs or a biotinylated goat anti-rabbit Ab (7.5 μg/ml; Vector) for cells incubated with hPL Ab, for 1 hour at room temperature. Slides were then washed and endogenous peroxidase activity was blocked by incubating the slides in 3% H_2O_2 in 10% methanol solution for 10 minutes and washed in PBS. Immunostaining was detected with the avidin-biotin-complex (ABC) peroxidase technique (Vector Laboratories; or StrAviGen Super-sensitive Concentrated Detection System, Biogenex, for slides incubated with anti-PCNA Ab) with diaminobenzidine (DAB; Sigma) as the chromogen. Negative controls were provided by incubating cells with an Ab of the same isotype (H-2K^k) for NDOG5 and PCNA or incubating cells with normal rabbit serum for hPL immunohistochemistry.

3. IMMUNOHISTOCHEMICAL STAINING FOR TGFß, AMPHIREGULIN, AND DECORIN IN THE HUMAN PLACENTA AND THE DECIDUA THROUGHOUT GESTATION

3.1. Tissues

Human placenta and decidua were retrieved from 7 different stages of pregnancy (n = number of placenta). Early gestational chorionic villi and decidua were obtained from elective terminations of pregnancies (11 and 14 weeks gestation; TGFß n=6; AR n=4; decorin n=5). Tissues, inclusive of chorionic villi and decidua, from later stages of gestational ages, 18 weeks (TGFß n=2; AR n=3; decorin n=3), 23 weeks (TGFß n=2; AR n=3; decorin n=3), 28 weeks (TGFß n=2; AR n=3; decorin n=3), 34 weeks (TGFß n=2; Ak n=2; decorin n=2), and term (TGFß n=5; AR n=3; decorin n=4), were collected from spontaneous preterm or term vaginal deliveries. All placentae were considered normal in size for gestational age and free of maternal or placental disease. Tissues were washed in PBS, fixed in 4% paraformaldehyde/0.2% glutaraldehyde, embedded in paraffin, and 5 μm thick sections cut with a microtome.

3.2. Immunohistochemistry

3.2.1. TGFß

Tissue sections were deparaffinized in xylene and rehydrated in decreasing ethanol concentrations and washed in PBS. Endogenous peroxidase activity was blocked with

H_2O_2 as described earlier in section 2.1.3. Tissue sections from all gestational ages were then treated with the antibody (Ab) 1D11.16.8, a mouse monoclonal antibody capable of recognizing TGFß$_1$ and TGFß$_2$ (8 μg/ml; kindly provided by Dr. H. Higley, Celtrix Laboratories, Palo Alto, CA), followed by the secondary Ab a biotinylated horse anti-mouse Ab (7.5 μg/ml; Vector Laboratory Inc., Burlingame CA). Tissue sections from both first trimester and term pregnancies were also treated with Ab CL-B1/29, a rabbit polyclonal Ab capable of recognizing TGFß$_2$ (125 μg/ml; kindly provided by Dr. H. Higley, Celtrix Laboratories). This was followed by the secondary Ab, a biotinylated goat anti-rabbit Ab (7.5 μg/ml; Vector Laboratory Inc., Burlingame CA). Immunostaining for all sections was then completed using the ABC-peroxidase technique (Vectastain ABC kit, Vector Laboratories Inc., Burlingame CA) with DAB as the chromogen. Subsequently the sections were counterstained with haematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Toronto ON).

Immunostaining specificity was confirmed by the appropriate negative controls. For Ab 1D11.16.8, negative controls were sections incubated in the absence of primary Ab and sections incubated in the presence of an unrelated anti-H-2K^k Ab of identical Ig isotype. For Ab CL-B1/29, negative controls were sections incubated without primary Ab, sections incubated with normal rabbit serum, and sections treated with Ab immunoabsorbed with purified TGFß$_2$.

3.2.2. Amphiregulin

Tissues sections were deparaffinized in xylene, rehydrated in decreasing

concentrations of ethanol, and washed in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked as described earlier. Sections were then treated with 10% normal goat serum in PBS for 45 min followed by the primary Ab, a rabbit polyclonal Ab AR-Ab1 (Johnson et al., 1991, 1992) at a concentration of 10 μg/ml in PBS inclusive of 0.1% BSA, and incubated overnight at 4°C. On the following day, slides were washed and the secondary Ab, a biotinylated goat anti-rabbit Ab (7.5 μg/ml; Vector Labs, Burlingame, CA) was applied for 1 h at room temperature. Positive immunoreactivity was visualized using the ABC-peroxidase technique (Vectastain ABC kit; Vector Labs) with DAB as the chromogen. The tissue sections were subsequently counter-stained with Carazzi's hematoxylin, dehydrated and mounted with Permount (Fisher Scientific, Toronto, ON).

The specificity of the immunostaining was confirmed with two negative controls in which the primary Ab was replaced with: 1) rabbit preimmune control IgG and 2) immunoabsorbed Ab (1 μg AR peptide per 1 μg Ab).

3.2.3. Decorin

Tissue sections were deparaffinized and endogenous peroxidase activity was neutralized as described above. Sections were incubated with 10% normal horse serum in PBS for 1 hour at room temperature and subsequently washed in PBS. The anti-decorin Ab, 6D6 a mouse monoclonal Ab (Pringle et al., 1985) at a concentration of 10 μg/ml was applied overnight at 4°C. The next day tissue sections were washed in PBS, incubated with a biotinylated horse anti-mouse Ab (7.5 μg/ml; Vector Labs) for 1 hour,

and washed in PBS. Immunoreactive decorin was detected with the ABC-peroxidase technique (Vector Labs) with DAB as the chromogen.

Negative controls were provided by following the same protocol except replacing the primary Ab with a non-specific anti-H-2K^k Ab of identical Ig isotype.

3.2.4. Cytokeratin Immunostaining

Extravillous cytotrophoblast cells embedded in the decidua were positively identified by routinely staining serial or semi-serial sections with an anti-cytokeratin mouse monoclonal Ab (CAM 5.2, Becton Dickinson, San Jose CA) following the same immunostaining procedure as outlined for Ab 1D11.16.8.

4. PROLIFERATION ASSAYS

4.1. Tritiated-Thymidine Incorporation Assay

To examine the effects of the growth factors (TGFα, EGF, AR, IGF-II, CSF-1) or their neutralizing or blocking antibodies (anti-TGFα, anti-EGF-receptor blocking Ab, anti-CSF-1) on early-passage (2-4) first trimester human trophoblast cell proliferation, as well as the effect of TGFα, EGF, and CSF-1 on choriocarcinoma cell proliferation, cells were cultured in RPMI complete medium for 24 hours in 96-well microtiter plates (Flow Laboratories, McLean, VA) at a concentration of 10^4 cells/well. Growth factors,

antibodies, or a combination of both (see below) were then added in triplicate or quadruplicate to the wells for 18 hours prior to exposure with ^{3}H-TdR (1 μCi/ml) for 6 hours. Following the total 24 hour incubation period, the medium was removed and 100 μl of 0.25% trypsin was added to each well for 15 min. Cells were subsequently harvested with a Titertek cell harvester and ß-counts were taken with a Beckman (Palo Alto, CA) scintillation counter.

Experiments done with antibodies alone were to test the effects of endogenous growth factors, and the combination of growth factor with their specific antibodies were to test the specificity of growth factor effects.

4.1.1. The Effect of TGFα on First Trimester Trophoblast Proliferation In Vitro

To investigate the effects of TGFα on trophoblast cell proliferation, various concentrations of TGFα ranging from 0-100 ng/ml were added in triplicates to the wells. In two other experiments, cells were treated in quadruplicate with TGFα (25 ng/ml in experiment 1 or 10 ng/ml in experiment 2), anti-TGFα neutralizing Ab (25 μg/ml; TAb-1, a gift from Dr. B. Langton, Berlex Biosciences, Alamada, CA), or with no additive. In one other experiment ^{3}H-TdR incorporation was measured as above in cells treated in quadruplicate with either no additive or TGFα (10 ng/ml) plus increasing concentrations (2.5, 5, 10, 20, and 40 μg/ml) of anti-TGFα neutralizing Ab (TAb-1).

In another experiment, cells were incubated with TGFα (10 ng/ml; Penninsula

Laboratories, Belmont, CA), anti-TGFα neutralizing Ab (25 μg/ml), anti-EGF-receptor blocking Ab (25 μg/ml; Upstate Biological Inc., Lake Placid, NY) or a combination of TGFα (10 ng/ml) plus anti-EGF-receptor blocking Ab (25 μg/ml).

4.1.2. The Effects of AR on First Trimester Trophoblast Proliferation In Vitro

To determine the effects of AR on trophoblast proliferation, AR was added at concentrations of 0.0, 1.0, 2.5, 5.0, 10, 25, 50, and 100 ng/ml diluted in serum free medium (Excell 300; JRH Biosciences, Lenexa, KS) in quadruplicate wells. No neutralizing Abs are yet available for AR thus no antibody experiments were performed.

4.1.3. The Effects of EGF and anti-EGF-receptor Blocking Antibody on First Trimester Trophoblast Proliferation In Vitro

To examine the effects of EGF on trophoblast cell proliferation, quadruplicate wells were incubated for 24 hrs with either EGF (10 ng/ml; Upstate Biological Inc. Lake Placid, NY), anti-EGF-receptor blocking Ab (25 μg/ml; Upstate Biological Inc.), or a combination of EGF plus anti-EGF-receptor Ab at the above concentrations.

4.1.4. The Effects of IGF-II on First Trimester Trophoblast Cell Proliferation In Vitro

To investigate the effects of IGF-II on first trimester trophoblast proliferation, IGF-II (Bachem California, Torrance, CA) was added at concentrations, 1.0, 2.5, 5.0,

10, 25, 50, and 100 ng/ml in quadruplicate wells.

4.1.5. The Effects of CSF-1 on First Trimester Trophoblast Cell Proliferation In Vitro

To examine the effects of CSF-1 on trophoblast growth, quadruplicate wells were incubated for 24h with either CSF-1 (10 ng/ml; Genzyme, Cambridge, MA), anti-CSF-1 neutralizing Ab (25 μg/ml; Oncogene Science, Uniondale, NY), no additive, or a combination of CSF-1 (10 ng/ml) plus increasing concentrations of anti-CSF-1 neutralizing Ab (25 μg/ml).

4.1.6. The Effects of EGF, TGFα, and CSF-1 on JAR, JEG, and BeWo Choriocarcinoma Cell Proliferation

To determine whether malignant trophoblast cells respond in similar manners to the growth factors the human choriocarcinoma cell lines, JAR, JEG3, and BeWo, were plated at 10^4 cells per well, in quadruplicate wells, in the presence of either TGFα, EGF, CSF-1, (all at 10 ng/ml), anti-TGFα neutralizing Ab (25 μg/ml), or no additive for 18 hours prior to the addition of ^{3}H-TdR. Six hours later the cells were harvested and ß-counts taken.

4.2. The Effects of TGFα and EGF on Proliferating Cell Nuclear Antigen (PCNA) Expression

Proliferative responses of human first trimester trophoblast cells treated with

TGFα and EGF were also examined by immunostaining for proliferating cell nuclear antigen (PCNA). Early passage first trimester trophoblast cells were cultured for 24h on chamber slides (Tissue-Tek, Nunc, Naperville, IL) at a concentration of 10^4 cells/chamber. Cells were treated in quadruplicate with either no additive or 10 ng/ml TGFα or EGF. Following incubation cells were fixed in ice-cold absolute methanol for 10 min at -20°C and then washed in PBS. To examine the incidence of cell nuclei expressing PCNA, immunostaining for PCNA was carried out as described earlier in section 2.1.3.

5. THE EFFECT OF TGFα ON MULTINUCLEATE CELL FORMATION BY FIRST TRIMESTER TROPHOBLAST

To determine if TGFα had an effect on the formation of multinucleated cells, $3x10^4$ first trimester trophoblast cells were plated in 6-well flat bottom Falcon plates (Becton Dickinson, San Jose, CA) for 24h. Cells were incubated in quadruplicate with TGFα (10 ng/ml), anti-TGFα neutralizing Ab (25 μg/ml), or no additive for 72h after which they were fixed in methanol for 3 min at -20°C and stained with haematoxylin. A total of 5000 cells and their nuclei were counted from each condition to determine the incidence of uninucleate and multinucleate cells.

6. IN VITRO MATRIGEL INVASION ASSAY

To examine the role of TGFα (Pennisula Laboratories), EGF (Upstate

Biotechnology Inc.), IGF-II (Bachem California), IGFBP-1 (Bachem California), and CSF-1 (Genzyme) on first trimester human trophoblast cell invasiveness, as well as the effect of TGFα on JAR choriocarcinoma cell invasiveness, a Matrigel invasion assay (Repesh, 1989) as modified by Graham et al (1993a) was performed. Briefly, 200 μl of a 600 μl/ml solution of Matrigel (Collaborative Research Inc. Bedford, MA) in cold RPMI 1640 medium was placed on 6.5 mm diameter Transwell filters with a pore size of 8 μm (Costar Corp., Toronto, ON). The Matrigel was then left to air-dry for 8 hrs in a laminar flow hood. Subconfluent cultures of second passage first trimester trophoblast cells were incubated in the presence of 10 μCi/ml ^{3}H-TdR in RPMI 1640 plus 10% FCS for 72 hrs. Cells were then trypsinized, washed, resuspended in medium, and the number adjusted to 2.5×10^5 cells/ml. A 200 μl sample of the cell suspension in the presence of growth factor, neutralizing Ab, binding protein (see below) or no additive was placed into quadruplicate upper wells of the Transwell chambers. The lower chambers received 800 μl of complete medium. After a 72 hr incubation period, the media in the upper and lower wells were removed and placed in separate tubes. Upper wells were washed once with PBS and the washing pooled with the media removed from the upper well. To remove cells adhering in the lower well and cells adhering to the underside of the filter, 800 μl of a 0.05% trypsin solution in PBS was placed in the lower well with the upper chamber reinserted for 20 min at room temperature. The trypsin solution was then removed and the lower wells washed once with 800 μl of PBS and pooled with the incubation media from the lower wells. Finally, each membrane was removed from the transwells with the aid of a scalpel and placed into separate tubes. Scintillation fluid was then added to each of the 3 tubes from each transwell and ß-counts

were taken with a Beckman scintillation counter. The invasion index was calculated as the amount of radioactivity in the lower wells expressed as a percentage of the sum of the total radioactivity (radioactivity from both wells plus the membrane).

Experimental Conditions for Invasion Assays (for first trimester trophoblast cells 1-8; for choriocarcinoma cells 9):

(1) TGFα 10 ng/ml in complete media (CM; RPMI 1640 + 10% FCS).

(2) Anti-TGFα 25 μg/ml in CM.

(3) EGF 10 ng/ml in CM.

(4) CSF-1 10 ng/ml in CM.

(5) Increasing doses of IGF-II, 0, 10, 50, 100, 250, and 600 ng/ml in CM.

(6) Increasing doses of IGF-II, 0, 10, 50, 100, 250, and 600 ng/ml in serum reduced media (SRM; RPMI 1640 + 1% FCS).

(7) Increasing doses of IGF-II, 0, 10, 50, 100, and 250 ng/ml in serum free media (SFM; RPMI 1640).

(8) Performed in SRM with:

a) no additive (control)

b) 150 ng/ml (20 nM) IGF-II

c) increasing doses of IGFBP-1, 0.1, 0.5, 1.0, 5.0, 10, 20, and 40 nM

d) combination of IGF-II (150 ng/ml) plus increasing doses of IGFBP-1, 0.1, 0.5, 1.0, 5.0, 10, 20, and 40 nM.

(9) JAR and JEG3 choriocarcinoma cells treated with 10 ng/ml TGFα, 25 μg/ml anti-TGFα neutralizing Ab and no additive.

7. RNA ISOLATION FOR NORTHERN BLOTTING

Duplicate flasks of early passage trophoblast cells at subconfluent density were cultured overnight in the presence of EGF (10 ng/ml), TGFα (10 ng/ml), anti-TGFα neutralizing Ab (25 μg/ml), CSF-1 (10 ng/ml), or no additive in complete media (RPMI 1640, 10% FCS) prior to RNA isolation. Total cellular RNA was isolated by the urea-lithium chloride-heparin technique described by Auffray and Rougeon (1980). Briefly, the cell culture media was discarded from the tissue culture flasks and 2 ml of fresh lithium chloride (LiCl)/urea/heparin solution was added. The solution was removed, homogenized with a polytron, and stored at 4°C for 16 hours. Samples were centrifuged at 12,000 g at 4°C for 30 min and the pellet was resuspended in 1 ml of LiCl/urea/heparin solution and stored for 1 hour at 4°C. After 1 hour the solution was centrifuged as described above and the pellet was resuspended in 500 μl TSH and stored for 1 hour at room temperature. Proteins were removed by phenol/chloroform extraction and the RNA was precipitated with 50 μl of 3M NaOAc and 1 ml 95% EtOH overnight at -20°C. The next day, samples were spun down and washed with 70% EtOH and resuspended in 500 μl DEPC treated H_2O. The concentration of total RNA was determined by optical density (O.D.) reading at 260 and 280 nm.

8. PLASMID PREPARATION FOR cDNA PROBES

E. coli grown in 250 ml LB broth and 500 μl ampicillin was inoculated overnight with the appropriate plasmid. The next day, the bacteria was centrifuged for 10 min at

3,000 g, the pellet resuspended in 9 ml resuspension buffer (25 mM Tris-HCl, pH 8.2, 10 mM EDTA, 50 mM glucose), and 1 ml of lysozyme solution (10 mg/ml) was added and the contents gently mixed and placed on ice for 5 min. Next, 20 ml of lysing solution (0.2% NaOH, 1% SDS) was added and the entire solution was mixed and left at room temperature for 5 min. Fifteen ml of 3.0 M potassium acetate, pH 5.0, was added and the solution placed on ice for 20 min prior to centrifugation for 10 min at 5,000 g. The supernatant was ethanol precipitated (95% ethanol) for 30 min on ice and subsequently, centrifuged for 30 min at 4,500 rpm. The pellet was dried, resuspended in 2 ml TE pH 8.0, and then 500 μl of 10.0 M ammonium acetate pH 5.0 was added to the plasmid DNA-containing material and incubated for 15 min on ice prior to centrifugation for 10 min at 8,500 rpm to precipitate high molecular weight RNA. The supernatant was phenol-chloroform extracted and the aqueous layer was precipitated with ethanol for 30 min on ice prior to centrifugation at 8,500 rpm at 4°C for 20 min. The pellet was resuspended in 900 μl of TE pH 8.0 and 10 μl of boiled RNAse A was added and this was incubated for 15 min at room temperature. Another phenol-chloroform extraction was performed and the aqueous layer ethanol precipitated. The sample was again spun down and the resulting DNA pellet was resuspended in 500 μl TE pH 8.0 and stored at -20°C.

To ensure a particular cDNA sequence was incorporated into a plasmid, the DNA was cut with appropriate restriction enzymes (GIBCO BRL). The resulting DNA fragments along with molecular weight markers were run on an agarose gel containing ethidium bromide following the procedure of Sambrook et al (1989). The gel was subsequently photographed and the resultant bands from the fragments were examined

to ensure they were of proper length.

9. NORTHERN BLOT ANALYSIS OF THE mRNA LEVELS OF THE INVASION ASSOCIATED MOLECULES (TIMP-1, TIMP-2, 72 kDa and 92 kDa type IV collagenase)

Five microgram samples of total RNA from each condition were electrophoresed through a 0.8% agarose/formaldehyde gel under denaturing conditions (Lehrach et al., 1977). The RNA in the gel was then transferred to Nytran membranes (Scheicher & Schuell, Keene, NH) with the aid of a BioRad 785 vacuum blotter (BioRad Laboratories, Mississauga, ON). cDNA probes were labeled with ^{32}P-αdCTP (50 μCi; 3000 Ci/mmol; Amersham Canada, Oakvilie, ON) using a Megaprime labeling kit (Amersham Canada, Oakville, ON). Unincorporated label was removed by means of a Pharmacia Nick Column (Pharmacia Canada, Baie d'Urfe, PQ). The following cDNA probes were used: human TIMP-1 cDNA probe in a pUC9 plasmid, generously provided by Synergen Corporation (Boulder, CO); human TIMP-2 cDNA probe cloned in a pGEM1 plasmid (Stetler-Stevenson et al., 1989); human 72 kDa type IV collagenase cDNA probe (Levy et al., 1991); and human 92 kDa type IV collagenase cDNA probe (Wilhelm et al., 1989).

Prehybridizations, hybridizations and washings were all performed in a roller bottle oven at 42°C. Blots were prehybridized for 3 hrs with a solution containing 50% formamide, 5x Denhardt's solution (Denhardt, 1966), 0.5% sodium dodecyl sulfate (SDS), 5x SSPE (1x SSPE = 0.15 M NaCl, 10 mM monosodium phosphate, 1 mM

EDTA), and 50 μg/ml denatured salmon sperm DNA. Blots were then hybridized overnight with denatured probes in fresh prehybridization solution. The Nytran membranes were washed twice with 2x SSPE, 0.1% SDS for 15 min, followed by a final wash with 0.1x SSPE, 0.1% SDS for 30 min. Blots were wrapped in plastic wrap, exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 18 to 24 hrs (except for blots hybridized with 92 kDa type IV collagenase probe, which were exposed for 5 days) at -80°C. Subsequently membranes were stripped using a boiling solution of 10 mM Tris and 1 mM EDTA.

10. SUBSTRATE GEL ZYMOGRAPHY

Secretion of gelatinases, the 72 kDa and 92 kDa type IV collagenase enzymes, was determined by substrate gel zymography. Early passage first trimester human trophoblast cells were cultured in six-well tissue culture plates at a concentration of $1.5X10^5$ cells per well for 48 hours in the presence of TGFα (10 ng/ml), EGF (10 ng/ml), or no additive in serum free media (Ex-cell medium, JRH Bioscience, Lenexa, KS) in triplicate. Cell conditioned media was lyophilized and stored at -20°C. The lyophilized conditioned media was rehydrated with 500 μl of Ex-cell media and protein concentration was measured according to the procedure of Bradford (1976). Samples of conditioned media from each condition containing 200 ng of protein were mixed with 6X SDS sample buffer and loaded into the wells of an SDS/polyacrylamide gel containing 2 mg/ml gelatin. Polyacrylamide gel electrophoresis was carried out at a constant current of 20 mA. Gels were subsequently washed twice in a solution of 2.5% Triton

X-100 (Sigma, St. Louis, MO) for 15 min each, rinsed with water, and incubated in a solution of 50 mM TRIS and 5 mM $CaCl_2$ overnight. The next day, gels were stained with Coomassie brilliant blue R-250 in 10% acetic acid 40% methanol, destained in 10% acetic acid 40% methanol, and dried between sheets of cellophane on a slab gel drier.

11. THE EFFECTS OF TGFα, EGF, AND CSF-1 ON hCG PRODUCTION BY THE CHORIOCARCINOMA CELL LINES JAR AND JEG3

To examine the effects of TGFα, EGF, and CSF-1 on hCG production by the choriocarcinomas, JAR and JEG3, cells were incubated in 6-well tissue culture plates at a concentration of 10^6 per well and allowed to adhere overnight. The next day the above growth factors were added at a concentration of 10 ng/ml in 2 ml to triplicate wells, and cultures were incubated for 48 hours. One ml samples of conditioned media were assayed for hCG production at the Department of Nuclear Medicine, St. Joseph's Hospital, London, Ontario, with a microparticle enzyme immunoassay (Abbott Laboratories, IL). Briefly, total hCG was measured in the samples by incubating the samples with a alkaline phosphatase conjugated anti-hCG Ab. The resulting complex were then incubated with anti-hCG coated microparticles and an aliquot of this was placed in a glass fiber matrix. The matrix was washed and then incubated with 4-methylumbelliferyl phosphate and the resulting fluorescence was assessed. The minimum amount of hCG detected by this assay is 2.0 mIU/ml.

12. STATISTICS

To determine the significance of the treatment effects, analysis of variance (ANOVA) was performed. When significance was detected a Duncan's new multiple range test was used for group comparisons. Differences were considered significant at $p \leq 0.05$.

V. RESULTS

1. FURTHER CHARACTERIZATION OF FIRST TRIMESTER HUMAN TROPHOBLAST CELLS PROPAGATED IN VITRO

First trimester human trophoblast cells obtained and cultured according to the method of Graham et al (1992) have previously been characterized for a number of markers (Graham et al., 1992). Since there is no strictly specific trophoblast cell marker, the identification of the cells propagated in vitro is dependent upon their positivity for certain markers and negativity for other markers.

1.1. Electron Microscopic Characteristics of Migrant Trophoblast Cells From Chorionic Villus Explants in Primary Cultures

Electron microscopic observations of cells near the chorionic villus explant-matrix interface revealed both multinucleate and mononucleate cells. Multinucleate, syncytiotrophoblast-like cells had pale cytoplasm and mononucleate cells flattened arrays of RER, polymorphic nuclei and filopodia (Figures 5a+b). Intercellular contacts with desmosomes and associated tonofilaments were also seen in the mononucleate cells (Figure 5c).

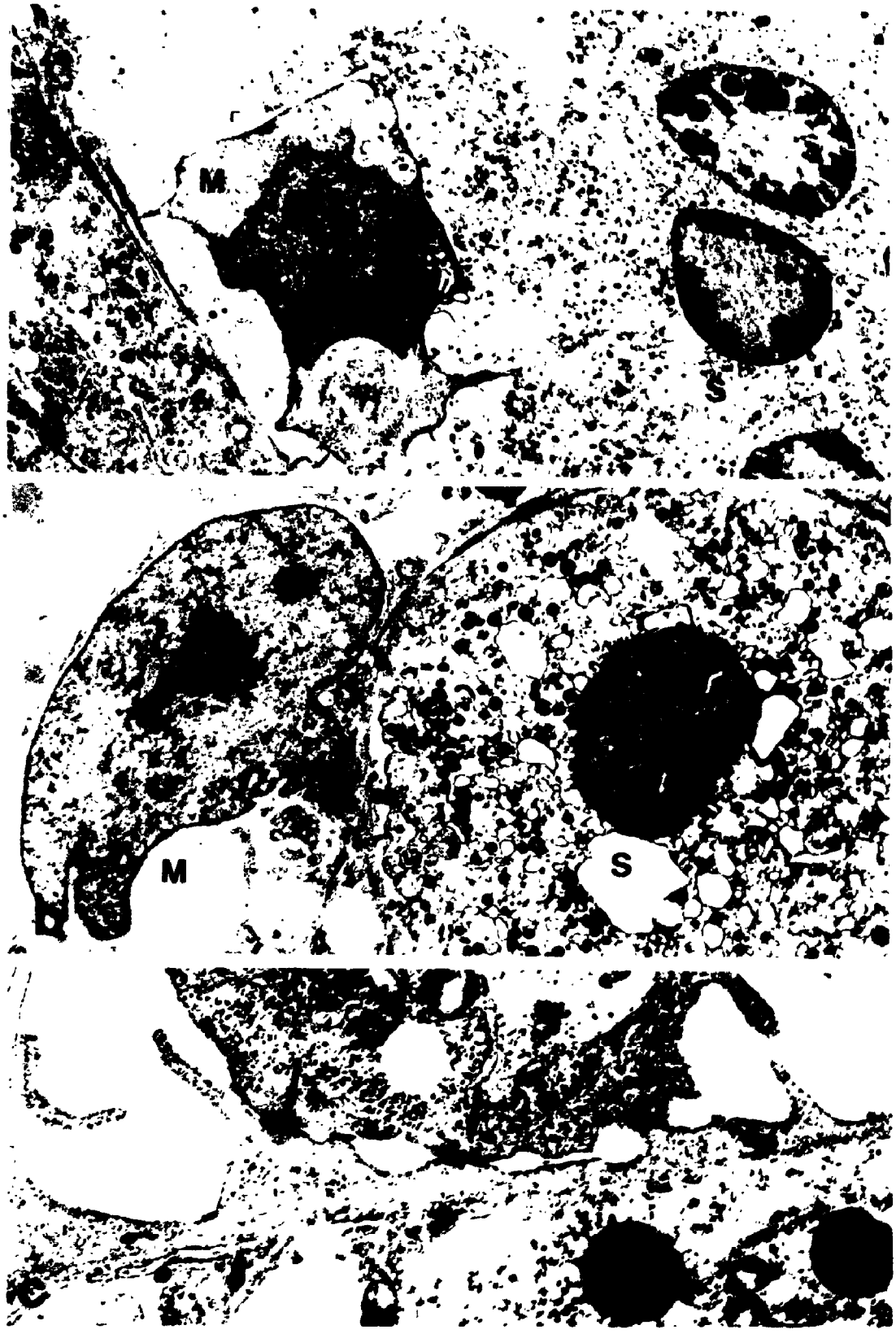
Electron micrographs of cells located deep in the collagen matrix displayed mononucleate cell characteristics, and the cells usually occupied neatly circumscribed cavities. These cells often had direct contacts with the matrix (Figure 6a). Small

Figure 5: Electron-photomicrograph of cells near the explant-matrix interphase.

a) Multinucleate syncytiotrophoblast-like cells (S) and mononucleate cell (M) . X3,300

b) Mononucleate cell (M) with flattened arrays of RER and section of a multinucleate syncytiotrophoblast-like cell (S). X5,600

c) Mononucleate cells with desmosomes (arrows). X17,280


M
S
M
S

filopodia were also often seen in smaller cavities within the matrix (Figure 6b).

1.2. Immuno-electron Microscopic Labeling of Migrant Cells From Primary Explant Cultures

Sections immuno-labeled for cytokeratin revealed specific and intense labeling (localization of gold particles) over intermediate filaments in both the multinucleate and mononucleate cells (Figure 7a). Cytokeratin labeling was also observed over tonofilaments associated with desmosomes. Such a desmosome located between two mononuclear cells is shown in figure 7b.

Specific immuno-labeling for hPL was noted only in the multinucleate cells, with no labeling in mononucleate cells. Such labeling was often seen over the RER of multinucleate cells (Figure 8).

Immunogold labeling for fibronectin showed abundant labeling over pericellular strands of fibrillar material and also over the contents of the RER within mononucleate cells (Figure 9a). Intracellular fibronectin labeling was absent from multinucleate cells. Immuno-labeling for oncofetal fibronectin showed a similar labeling pattern to that of fibronectin, being seen over pericytoplasmic strands of fibrils; however, no intracellular immuno-labeling was observed for oncofetal fibronectin (Figure 9b).

Figure 6: Electron-photomicrograph of cells within the collagen gel.

a) Cells which invaded the matrix occupied neatly circumscribed cavities and often had direct contact sites with the matrix (arrow). X6,626

b) Filopodia (F) were also seen in cavities in the matrix. X6,629

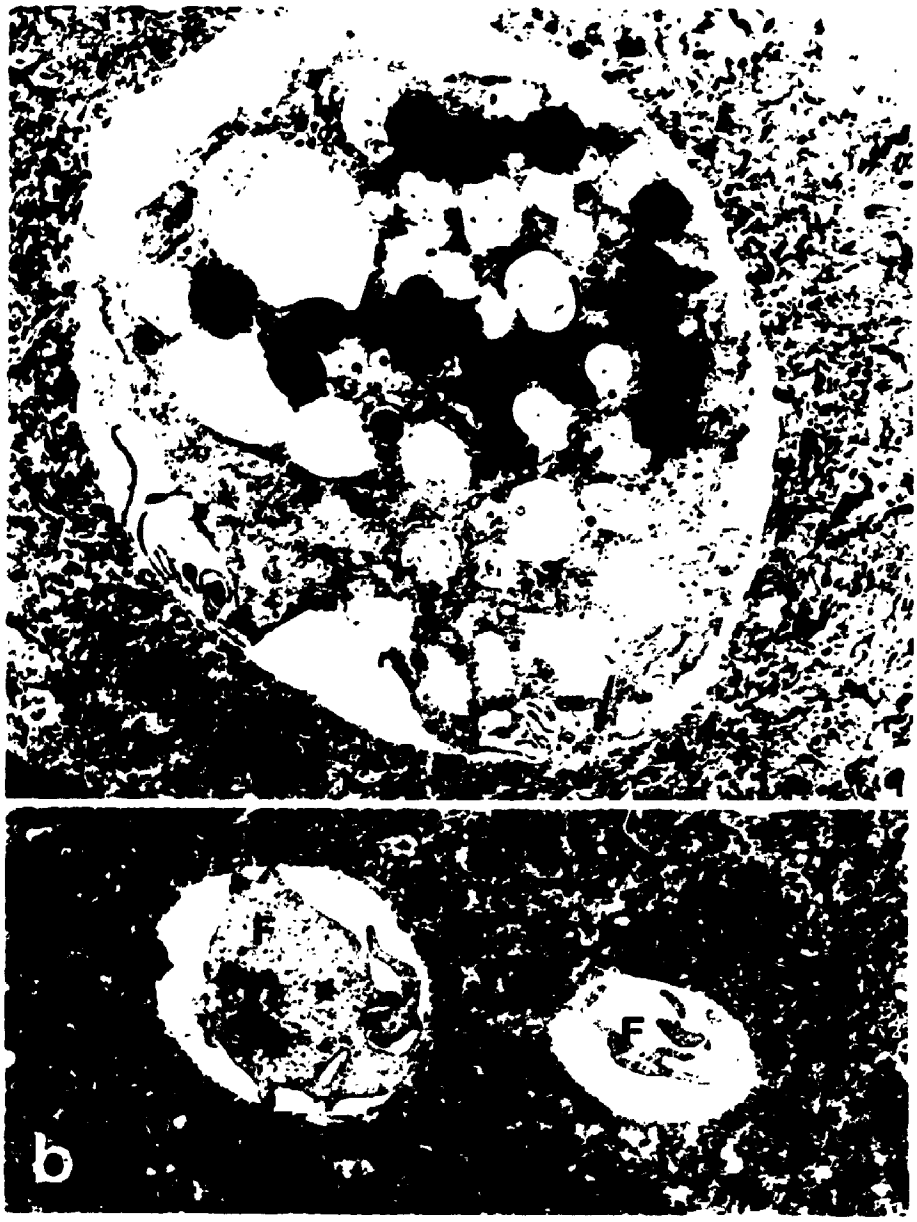

a
b
F
F

Figure 7: Electron-photomicrograph of migrant cells from primary explant grown on collagen gel and labeled with immunogold for cytokeratin.

a) Specific immuno-labeling for cytokeratin is observed in both mononucleate (closed arrow) and multinucleate (open arrow) cells. X12,420

b) Cytokeratin positive tonofilaments (T) associated with a desmosome (D) are also seen between two cells. X22,090


a
T
D
b

Figure 8: Electron-photomicrograph of migrant cells in primary explant cultures grown on collagen gel showing immunogold labeling for hPL. Specific labeling for hPL was seen only in multinucleate cells. This labeling was often seen over the RER (arrow). X17,940

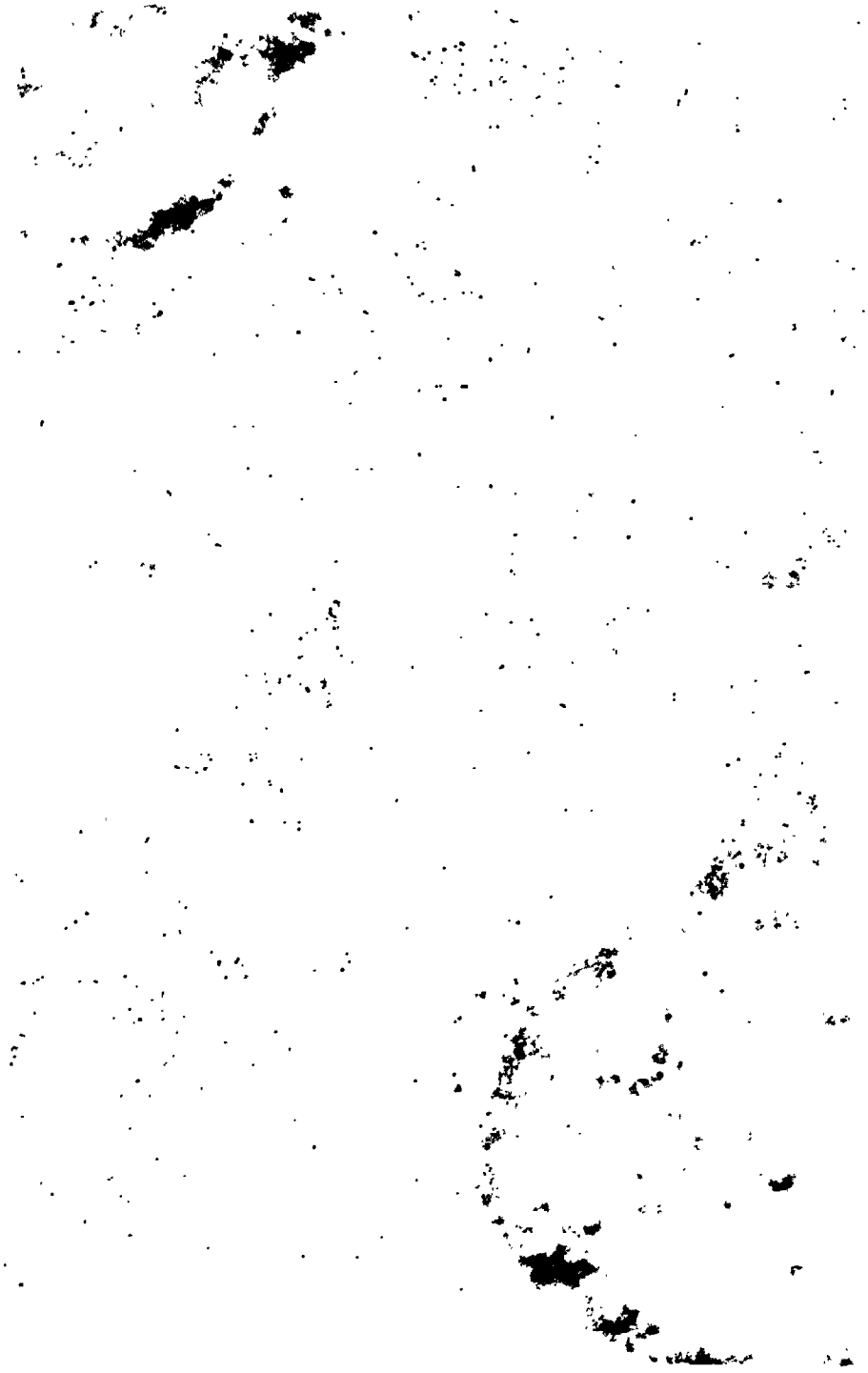
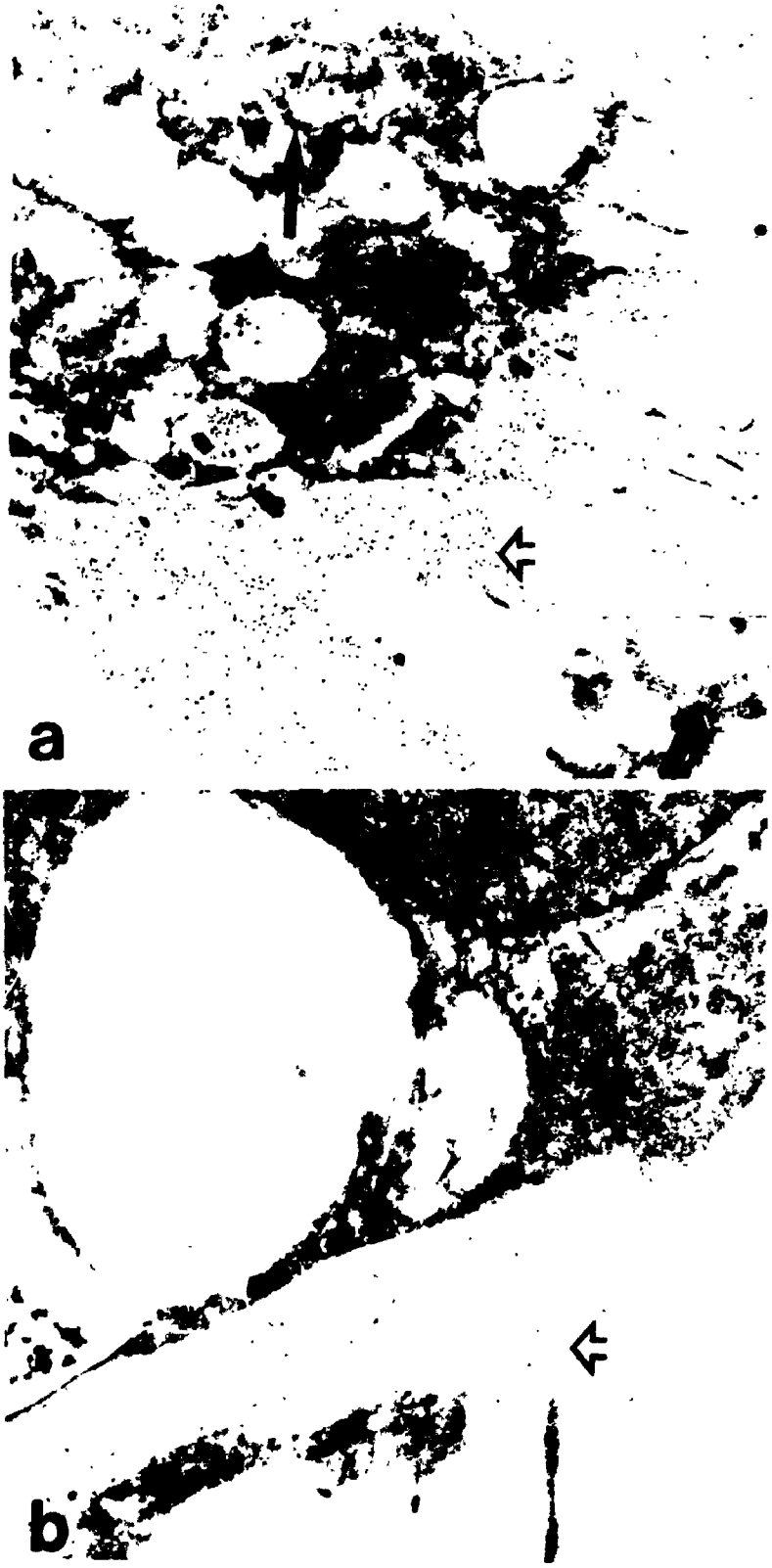


Figure 9: Electron-photomicrograph of cells from primary explant culture grown on collagen gel, subjected to immunogold labeling for (a) fibronectin and (b) oncofetal fibronectin.

a) Specific labeling for fibronectin was observed over pericellular strands of material (open arrow) and also over the REP (closed arrow, and in inset) within mononucleated cells. X9,180

b) Specific labeling for oncofetal fibronectin was also observed over pericytoplasmic strands of material (open arrow); however, no intracellular labeling was seen. X13,500


a
b

1.3. Immunophenotypic Characterization of First Trimester Human Trophoblast Cells Propagated In Vitro

To further characterize the first trimester human trophoblast cells propagated in vitro, cells were immunostained for NDOG5, hPL, and PCNA after 2-4 passages in vitro.

NDOG5 is an antibody reported to recognize human intermediate trophoblast cells (Shorter et al., 1993). Approximately 50% of the cells grown in vitro were immunoreactive with this Ab (Figure 10).

Immunoreactivity for hPL was observed in a minority of the cells in culture. The immunostaining was present only in the multinucleate cells with no immunostaining seen in the mononucleate cells (Figure 11).

PCNA, a marker for proliferating cells, was detected in less then 1% of the mononuclear cells passaged in culture (Figure 12a). Cells incubated with an Ab of similar Ig isotype (H-2K^k, negative control) revealed no immunostaining (Figure 12b).

2. IMMUNOLOCALIZATION OF TGFß, AR, AND DECORIN IN THE HUMAN PLACENTA AND THE DECIDUA THROUGHOUT GESTATION

To determine the geographic and temporal distribution of the growth factors, TGFß and AR, and the proteoglycan, decorin, immunohistochemistry was performed on tissue sections of human placenta and the decidua throughout gestation.

Figure 10: Photomicrograph of NDOG5 (a selective marker for extravillous trophoblast cells) immunolabeled trophoblast cells in culture. X140

Figure 11: Photomicrograph of hPL immunolabeled trophoblast cells in culture. Note only the multinucleate cells were immunoreactive for hPL. X280

Figure 12: Photomicrograph of PCNA immunolabeled trophoblast cells grown in culture and countered stained with haematoxylin.

a) PCNA immunoreactivity was observed only in a minority of mononucleate cells (arrow). X870

b) cells incubated with a nonspecific Ig isotype (H-2K^k), negative control. X870

Marker	Trophoblast labeled(% positive or +/-
cytokeratin[7]	>99%
IGF-II[7]	>90%
NDOG-5[6]	~50%
hPL[6]	multinucleate cells only
β_1 integrin[7]	>99%
vitronectin receptor[7]	>98%
α_1, α_3, α_5, α_v integrins[7]	93-98%
α_6, β_4 integrins[7]	0%
type IV collagenase[1,3,6]	+
hCG[2]	↓ with ↑ passages
uPA[4]	+
uPA receptor[5]	+
TIMP-1[3,4]	+
TIMP-2[6]	+
placental alkaline phosphatase[4]	+
PCNA[6]	0.7%
vimentin[2]	0% -slight reactivity with increasing passages
63D3[2,4] (macrophage marker)	-
factor VIII[4] (endothelial cell marker)	-

Table 1. Phenotypic profile of cultured first trimester human trophoblast cells (2-8 passages). 1, Yagel et al, 1988; 2, Yagel et al, 1989; 3, Graham and Lala, 1991; 4, Graham et al, 1992; 5, Zini et al, 1992; 6, this thesis; 7, Graham et al, 1993.

2.1. Immunolocalization of TGFß in the Human Placenta and the Decidua Throughout Gestation

Immunohistochemistry performed on tissue sections from first trimester tissue (6 subjects) using either the monoclonal (1D11.16.8) or the polyclonal (CL-B1/29) antibody and tissue sections from 18 weeks gestation employing the monoclonal Ab revealed strong localization of TGFß to the extracellular matrix (ECM) of the decidua with very few decidual cells exhibiting intracellular labeling. Leukocytes in the decidual tissue were negative for TGFß (Figure 13a). The uterine and glandular epithelium were unreactive for TGFß (not shown). Chorionic villi revealed TGFß immunostaining to the syncytiotrophoblast layer of the chorionic villi with moderate labeling of the villous core and occasional immunostaining of Hofbauer cells in the villous core. The villous cytotrophoblast cells were not immunostained (Figure 13b). Tissue sections incubated in the absence of primary Ab or with an unrelated Ab of similar Ig isotype to the monoclonal anti-TGFß Ab (negative controls for 1D11.16.8, the monoclonal Ab) demonstrated no labeling (Figure 13c). Negative controls for the polyclonal anti-TGFß Ab, CL-B1/29, sections incubated with normal rabbit serum or immunoabsorbed serum also displayed no immunoreactivity (data not shown). Immunostaining of first trimester tissue using both Abs revealed a similar labeling pattern.

Immunostaining for TGFß employing the monoclonal anti-TGFß antibody on tissue sections from 23 and 28 weeks of gestation demonstrated TGFß immunoreactivity associated with the ECM of the decidua and intracellular immunoreactivity in decidual cells (Figure 14a). Decidual leukocytes were again negative for TGFß. Uterine and

Figure 13: Representative pictures of immunolabeling for TGF-ß in human first trimester decidua and placenta. All sections were counterstained with haematoxylin.

a) Decidual tissue showed specific immunoreactivity for TGF-ß associated with the ECM. Few decidual cells exhibited intracellular immunostaining (closed arrow). Decidual leukocytes were negative (open arrow). X280

b) First trimester chorionic villi revealed TGF-ß immunoreactivity within the syncytiotrophoblast cell layer (ST) and moderate staining of the mesenchymal core of the villi. Villous cytotrophoblast cells (CT) were not immunoreactive. X700

c) A negative control of first trimester decidual tissue immunostained with an unrelated anti-H-2K^k Ab, a mouse monoclonal Ab of the same isotype (IgG2b). X140


ST
CT
b

glandular epithelium were negative for TGFß (not shown). Villous syncytiotrophoblast continued to display intracellular immunoreactivity and the mesenchymal core of the villi remained moderating stained. The villous cytotrophoblast cells were again negative for TGFß (Figure 14b). Extravillous cytotrophoblast cells embedded in the decidua (their identity confirmed by cytokeratin immunostaining in serial or semi-serial sections) whenever encountered in tissue sections exhibited intracellular immunoreactivity for TGFß (data not shown). Tissues sections incubated in the absence of primary Ab or in the presence of an unrelated Ab of similar Ig isotype were again negative (data not shown).

Tissue sections from 34 weeks (2 subjects) and term pregnancies (5 subjects) immunostained for TGFß revealed a marked decrease of immunostaining of the decidual ECM and by term very little to no staining was observed. The majority of decidual cells at these later gestational ages exhibited strong intracellular labeling. Migrant leukocytes embedded in the decidua remained negative for TGFß (Figure 15a). The syncytiotrophoblast layer of the chorionic villi continued to be immunoreactive for TGFß as well as the moderate staining of the villous core. Extravillous trophoblast cells of the cytotrophoblastic shell, identified by cytokeratin staining of serial or semi-serial sections, displayed TGFß positivity (Figure 15b). Tissue sections which served as negative controls were always negative (data not shown). Tissues from term pregnancies demonstrated identical immunostaining patterns following immunolabeling with either Ab.

In summary, TGFß immunostaining at early gestational ages was associated with the ECM of the decidua with few decidual cells exhibiting intracellular immunoreactivity;

Figure 14: Representative photomicrographs of sections of decidua and placenta from 23 weeks of gestation immunostained for TGF-ß. All sections were counterstained with haematoxlyin.

a) TGF-ß immunoreactivity is associated with the decidual ECM and few decidual cells (arrow) exhibit intracellular labeling. X140

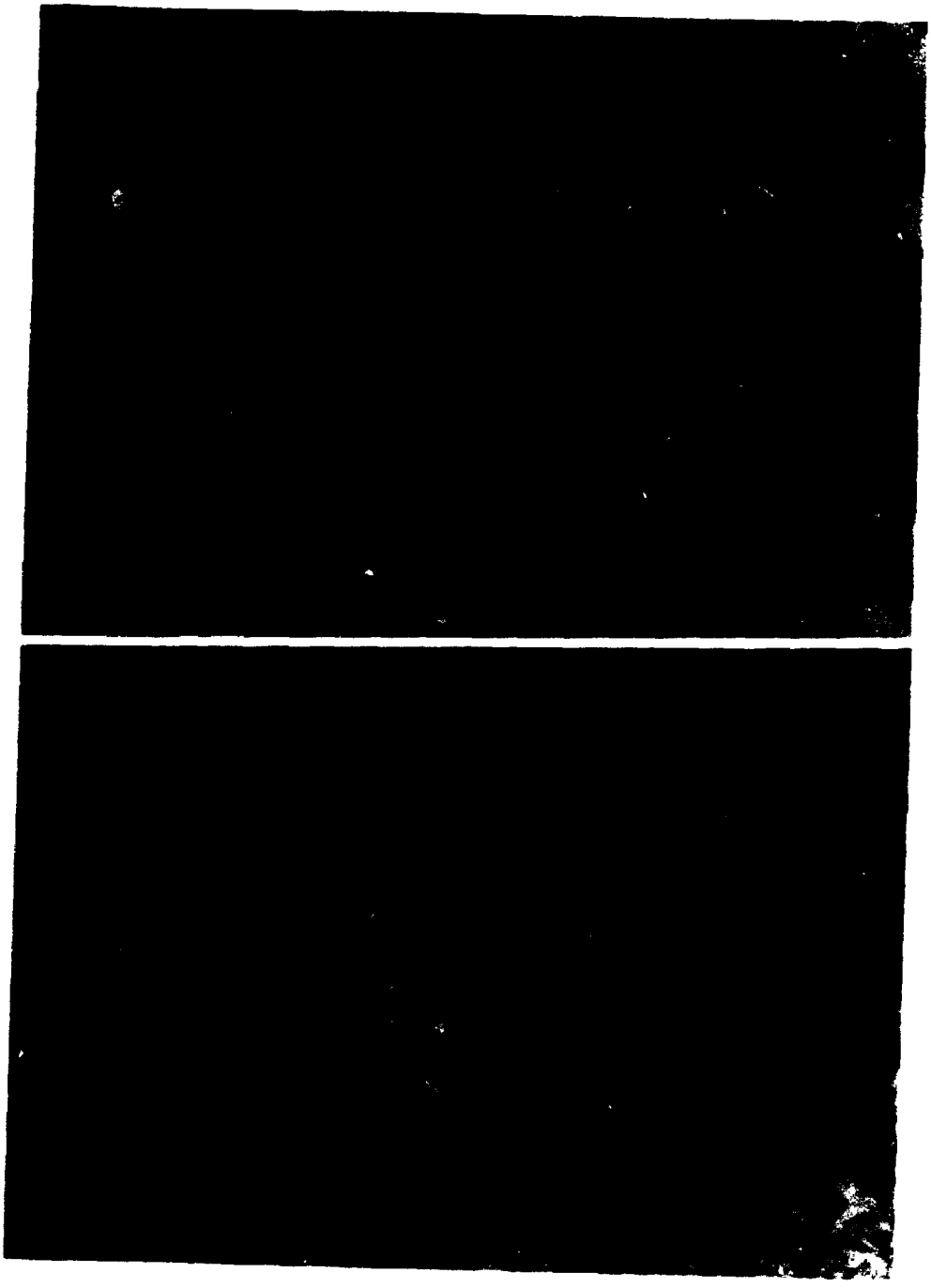
b) Chorionic villi from 23 weeks display TGF-ß immunostaining in the syncytiotrophoblast cell layer (ST), with moderate staining of the villous core, and no staining of the cytotrophoblast cell layer (arrow). X700

b

Figure 15: Photomicrographs of tissue sections of term decidua and placenta immunolabeled for TGF-ß and counterstained with haematoxylin.

a) Term decidual tissue reveals the majority of decidual cells are now positive for TGF-ß intracellularly, while TGF-ß immunoreactivity in the ECM is sparse. Migrant leukocytes were negative (arrow). X700

b) A tissue section at term containing decidua (D), extravillous trophoblast of the cytotrophoblastic shell (CS), and chorionic villi (CV) reveals TGF-ß immunoreactivity within decidual cells, extravillous trophoblast cells (their identity confirmed in serial sections immunostained for cytokeratin; not shown), and in the syncytiotrophoblast cell layer. X280



at later gestational ages this distribution shifted with increasingly more decidual cells displaying cytoplasmic labeling and less staining observed in the ECM, until at term when the majority of decidual cells showed cytoplasmic staining and no staining in the ECM. Decidual leukocytes remained negative throughout gestation. The villous syncytiotrophoblast cell layer was immunolabeled throughout gestation as well as the mesenchymal core of the villi. The occasional Hofbauer cell in the [illegible] of the chorionic villi was also labeled for TGFß. Extravillous trophoblast cells whenever encountered were immunoreactive for TGFß. Villous cytotrophoblast cells were consistently negative for TGFß.

2.2. Immunolocalization of Amphiregulin in the Human Placenta and the Decidua Throughout Gestation

Immunohistochemistry performed on tissue sections from 11, 14, and 18 weeks of gestation revealed strong immunostaining of AR to cytoplasm and nuclei of the syncytiotrophoblast cell layer of the chorionic villi in all cases. The villous cytotrophoblast cells and cells of the villous core showed no AR immunoreactivity (Figure 16a). Decidual cells, decidual leukocytes (Figure 16b) and uterine and glandular epithelial cells (data not shown) were also negative for AR. Tissue sections incubated in the presence of preimmune IgG or immnoabsorbed Ab showed no staining at all three gestational ages examined (Figure 16c).

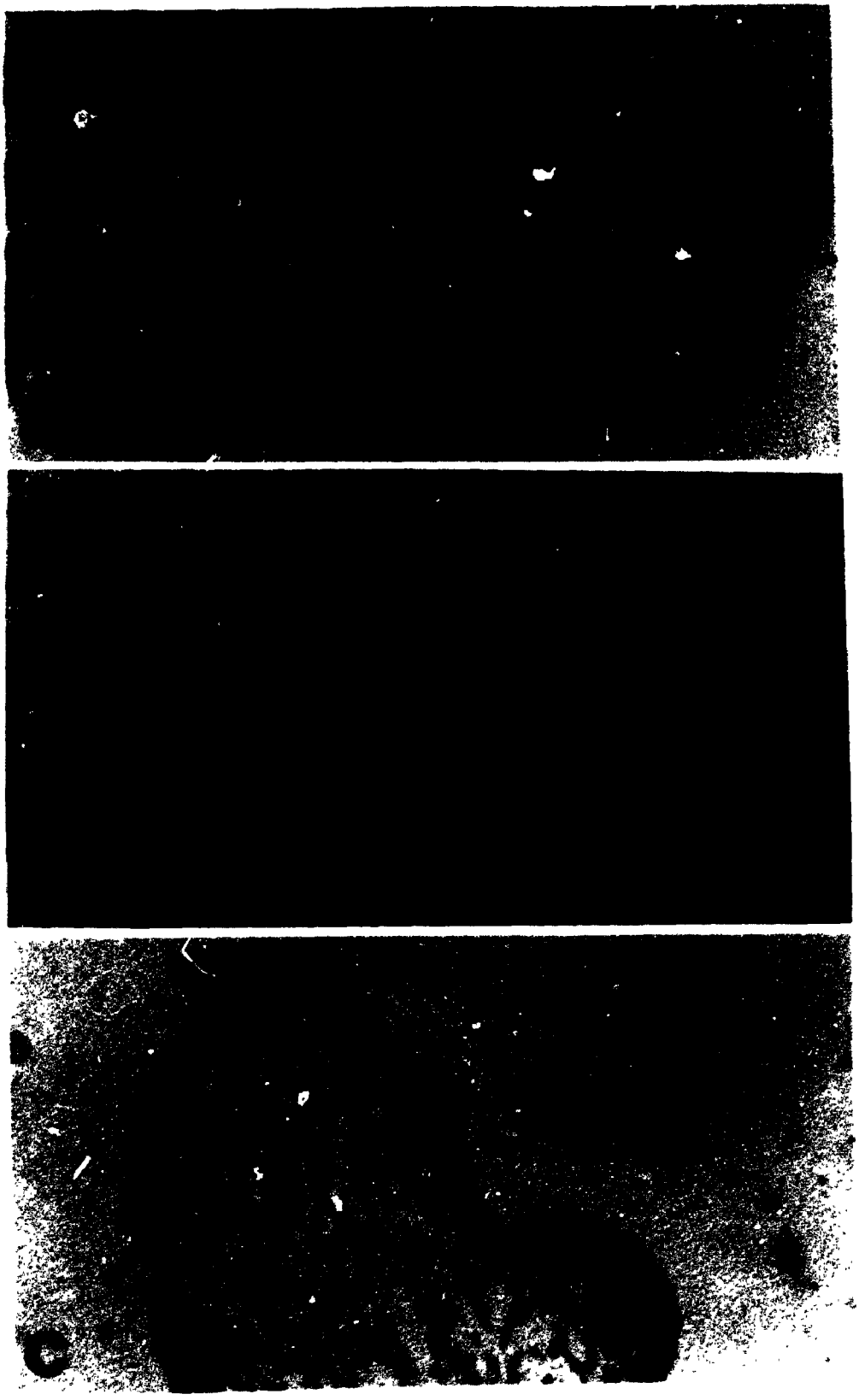
At later gestational ages (23, 28, 34 weeks, and term) AR immunoreactivity was absent from all tissues examined inclusive of the syncytiotrophoblast cell layer, villous

Figure 16: Photomicrographs of sections of early gestational placenta and decidua immunostained for AR and counterstained with haematoxylin.

a) Early gestational chorionic villi revealed specific AR immunoreactivity in the cytoplasm (open arrow) and the nuclei (closed arrow) of the syncytiotrophoblast cell layer. The villous cytotrophoblast and cells of the mesenchymal core show no AR immunoreactivity. X560

b) First trimester decidual tissue displayed no specific immunostaining. X220

c) No immunostaining was observed in first trimester chorionic villi when sections were incubated in the presence of immunoabsorbed AR. X220



cytotrophoblast cells, extravillous trophoblast cells embedded in the decidua, maternal decidual cells and immigrant leukocytes within the decidua (data not shown). Negative control tissue sections for the gestational ages examined showed no staining (data not shown).

In summary immunostaining for AR was confined to the syncytiotrophoblast cell layer, both cytoplasm and nuciei, in early pregnancy.

Table 1 summarizes the location of the growth factors in the human placenta and the decidua.

2.3. Immunolocalization of Decorin in the Human Placenta and the Decidua Throughout Gestation

Immunostaining of first trimester placental and decidual tissue for decorin revealed strong immunoreactivity localized to the ECM of the first trimester decidua, whereas decidual cells, decidual leukocytes, and the uterine epithelium were negative (Figure 17a). Sections of chorionic villi showed decorin immunoreactivity in the mesenchymal core of the villi and no staining of the syncytiotrophoblast or cytotrophoblast cell layers (Figure 17b). No immunoreactivity was observed in negative control sections (Figure 17c).

Tissue sections from 18, 23, 28, and 34 weeks of pregnancy showed similar immunolabeling patterns for decorin. In sections of decidual tissue, immunoreactivity was seen to decrease in the ECM and few decidual cells showed weak staining. Decidual leukocytes and uterine and glandular epithelium remained negative (data not shown).

Table 2 Summary of the Location of Growth Factors in the Human Placenta and the Decidua

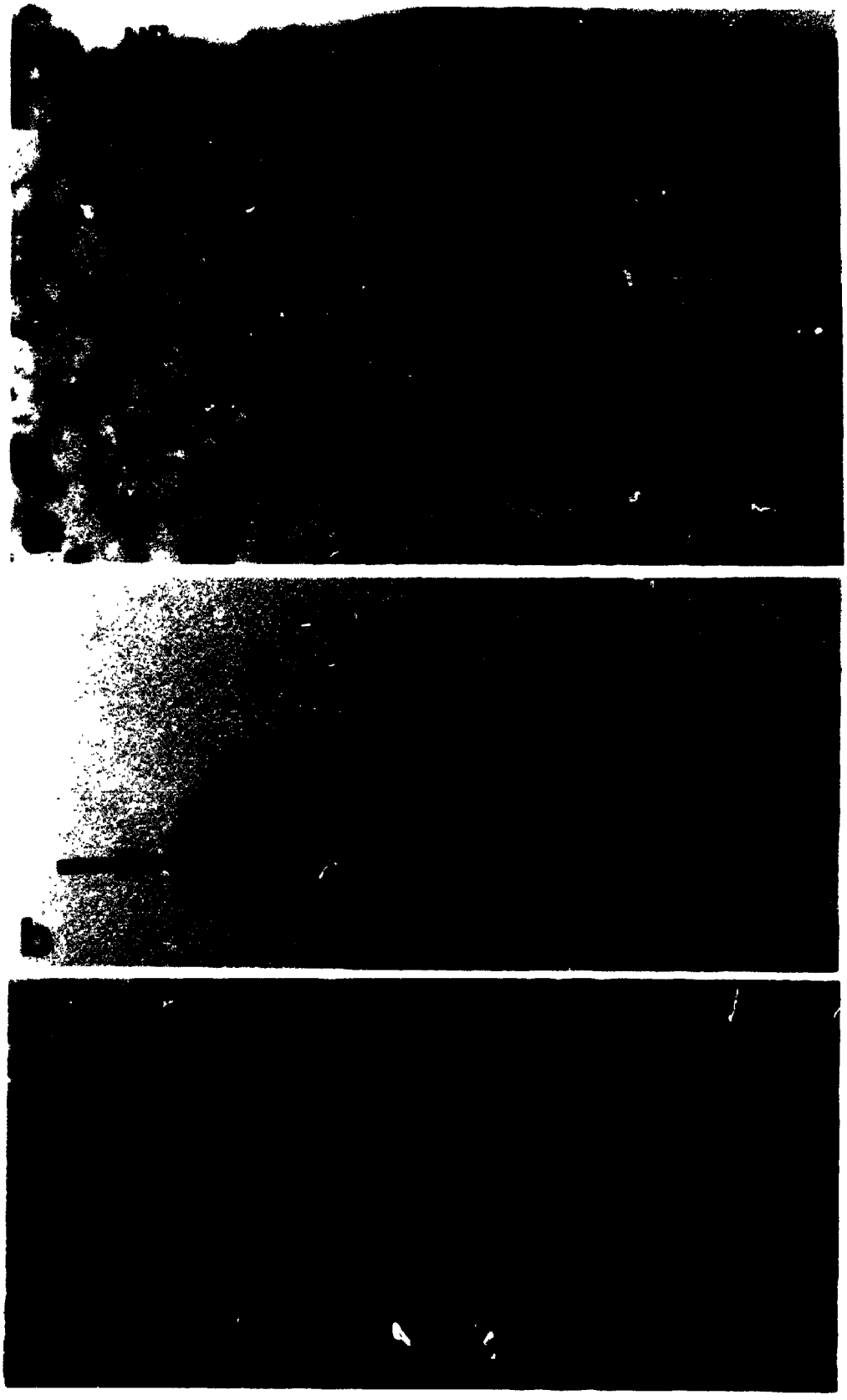
Growth Factor	Placenta	Decidua
EGF	Villous cytotrophoblast and syncytiotrophoblast (protein, Hofmann et al., 1991; Bissonnette et al., 1992)	Uterine epithelial and decidual cells (protein, Hofmann et al., 1991; and mRNA, Haining et al., 1991)
TGFα	Villous cytotrophoblast, syncytiotrophoblast, and intermediate trophoblast (protein, Lysiak et al., 1992)	Uterine epithelial and decidual cells (protein, Lysiak et al., 1992 and mRNA, Haining et al., 1991)
AR	Syncytiotrophoblast, until week 18 (protein; this thesis)	Not detected (this thesis)
TGFß	Syncytiotrophoblast (protein, this thesis; mRNA, J. Hunt, personal communication), intermediate trophoblast (protein, this thesis)	First trimester: primarily ECM, few decidual cells; later gestation: decidual cells (protein, this thesis)
CSF-1	mRNA in the placenta (Saji et al., 1990)	mRNA in the decidua (Saji et al., 1990)
IGF-II	All trophoblast subsets (protein); first trimester villous cytotrophoblast and intermediate trophoblast of all ages (mRNA, V.K.M. Han, personal communication)	Decidual cells (protein) (IGFBP-1 mRNA in decidual cells adjacent to the intermediate trophoblast; V.K.M. Han, personal communication)

Figure 17: Representative pictures of sections of first trimester placental and decidual tissue immunostained for decorin and counterstained with haematoxylin.

a) First trimester decidual tissue revealed decorin immunoreactivity localized to the decidual ECM with no staining of decidual cells (open arrow), decidual leukocytes (closed arrow), or the uterine epithelium (UE). X280

b) First trimester chorionic villi showed the mesenchymal core (MC) of the villi immunoreactive for decorin. Both trophoblastic layers of the villi were negative (arrow). X220

c) First trimester decidua (D) and chorionic villi (CV) incubated in the presence of H-2K^k (negative control). X220



The mesenchymal core of the chorionic villi continued to exhibit moderate labeling and both the syncytiotrophoblast and cytotrophoblast cell layers remained unreactive (data not shown).

Immunostaining of sections from term tissues revealed sparse decorin immunoreactivity in the ECM of term decidua with very few decidual cells displaying intracellular labeling. Decidual leukocytes and uterine and glandular epithelium remained negative (Figure 18a). Term chorionic villi continued to show immunostaining of the mesenchymal core and the syncytiotrophoblast cell layer remained negative (Figure 18b).

In summary, decorin was localized to the ECM of the decidua with the most intense labeling seen in the first trimester decidua, and few decidual cells exhibited weak immunoreactivity. The mesenchymal core of the chorionic villi displayed a moderate amount of decorin immunoreactivity throughout gestation.

3. THE EFFECTS OF THE GROWTH FACTORS ON THE PROLIFERATION OF FIRST TRIMESTER HUMAN TROPHOBLAST CELLS IN VITRO

3.1. Effect of the EGF-receptor Ligands on Trophoblast Cell Proliferation

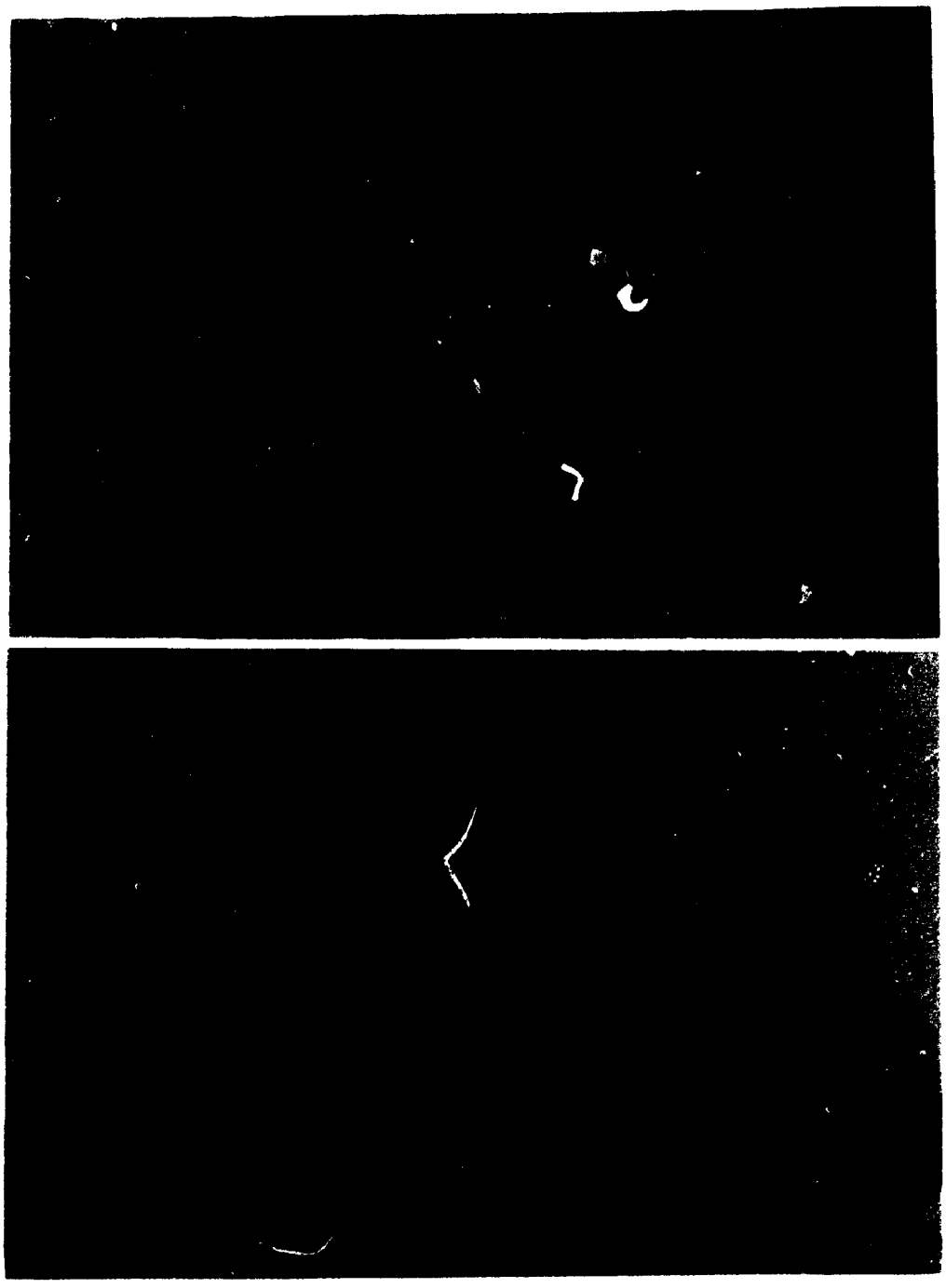
3.1.1. The Effect of TGFα and EGF on Trophoblast Cell Proliferation

The presence of increasing concentrations of TGFα to first trimester trophoblast cells in culture caused a dose dependent stimulation of proliferation which reached a plateau at 6-100 ng/ml; this plateau was slightly more than double the basal level (Figure 19). Figure 20 shows the results when first trimester trophoblast cells were incubated

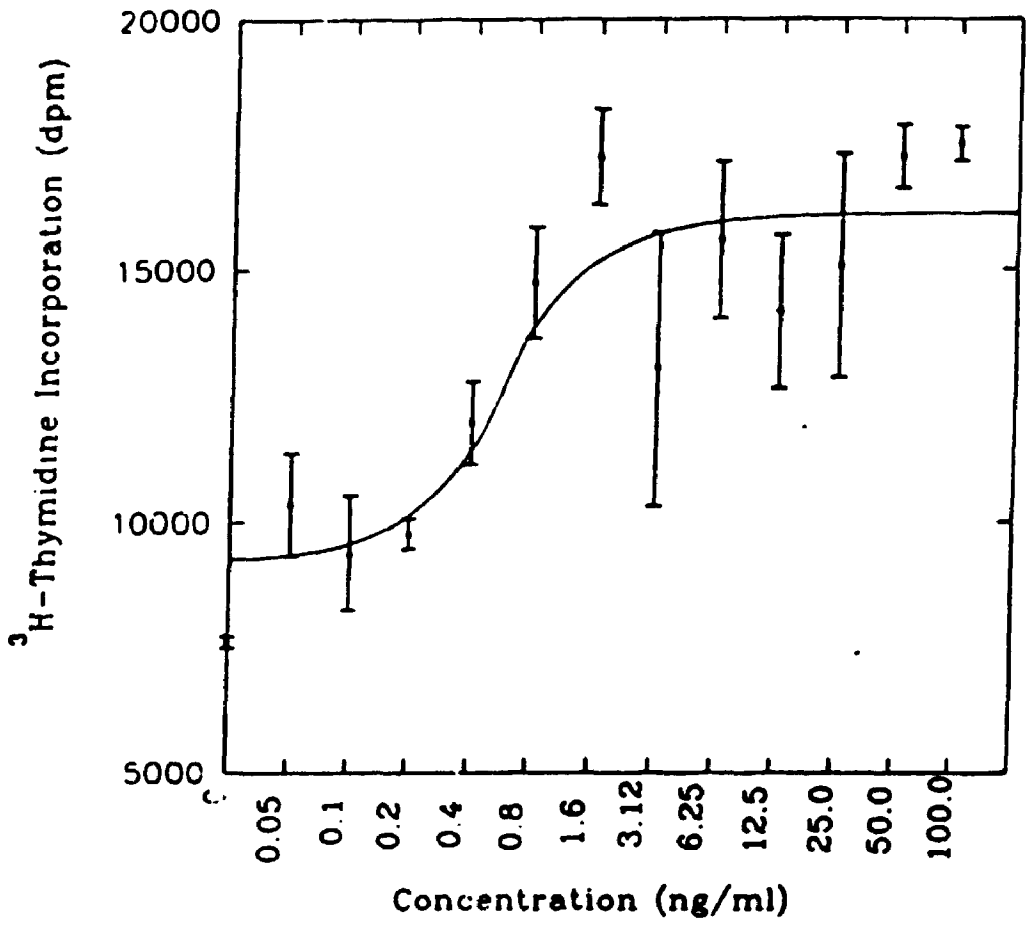
Figure 18: Photomicrographs of term placental and decidual tissue sections immunostained for decorin and counterstained with haematoxylin.

a) Light staining of the decidual ECM as well as some decidual cells (open arrow) is observed in term decidual tissue. X350

b) Term chorionic villi continued to display decorin immunoreactivity associated with the villous core (VC) only. X350



igure 19: The effects of various concentrations of TGF-α (0-100 ng/ml) on human first trimester trophoblast cell proliferation (^{3}H-TdR incorporation). Each bar represents the mean ($\pm$ SE) of triplicate wells. All data points at TGFα concentrations $\geq$ 0.8 ng/ml revealed a significant increase ($p < 0.05$) over control (0 ng/ml) values, exc pting the data point at 3.12 ng/ml.


20000
15000
10000
5000
^{3}H-Thymidine Incorporation (dpm)
0.05
0.1
0.2
0.4
0.8
1.6
3.12
6.25
12.5
25.0
50.0
100.0
Concentration (ng/ml)

with either no additive, neutralizing anti-TGFα Ab, or TGFα (25 ng/ml experiment 1, or 10 ng/ml experiment 2) for 24h. There was no significant difference in ^{3}H-TdR uptake between control cultures and cultures treated with neutralizing anti-TGFα Ab indicating the absence of significant levels of endogenous TGFα in these cultures. Cells incubated in the presence of TGFα again showed a significant increase in ^{3}H-TdR incorporation at both concentrations of TGFα used ($p < 0.01$) over control incubations. Cells cultured in the presence of TGFα (10 ng/ml) plus increasing concentrations of anti-TGFα neutralizing Ab (2.5, 5, 10, 20, and 40 μg/ml) showed an antibody dose-dependent decline in ^{3}H-TdR incorporation between 5 - 20 μg/ml to control levels at doses of 20 - 40 μg/ml (Figure 21). These results indicate that the neutralizing Ab was capable of abolishing the proliferative effects of exogenous TGFα and thus the absence of effects of the Ab at 25 μg/ml on ^{3}H-TdR incorporation by trophoblast cell (Figure 20) was not due to a lack of antibody function.

In another experiment, first trimester human trophoblast cells incubated in the presence of EGF (10 ng/ml) or TGFα (10 ng/ml) showed a significant increase in ^{3}H-TdR incorporation ($p < 0.01$) compared to cells incubated with no additive or anti-TGFα neutralizing Ab (25 μg/ml; Figure 22). There was no significant difference between control cultures and cells treated with anti-TGFα neutralizing Ab indicating the lack of significant endogenous TGFα in these cultures. Figure 23 shows the results when trophoblast cells were cultured in the presence of EGF (10 ng/ml), TGFα (10 ng/ml), anti-EGF-receptor blocking Ab (25 μg/ml), EGF plus anti-EGF-receptor Ab, TGFα plus anti-EGF-receptor Ab, or no additive. Trophoblast cells treated with EGF or TGFα again showed a significant increase in proliferation over controls levels ($p < 0.05$). The

Figure 20: Results from two ^{3}H-TdR incorporation assays with second passage first trimester human trophoblast cells. Addition of TGFα caused a significant ($p<0.01$ EXP#1 and $p<0.01$ EXP#2) increase in ^{3}H-TdR uptake. Control, complete media alone; TGF-α, 25 ng/ml in experiment #1 and 10 ng/ml in experiment #2; anti-TGF-α Ab, 25 μg/ml in both experiments. A star (*) in all subsequent legends represents data points significantly ($p < 0.05$) different from the values obtained under control conditions.

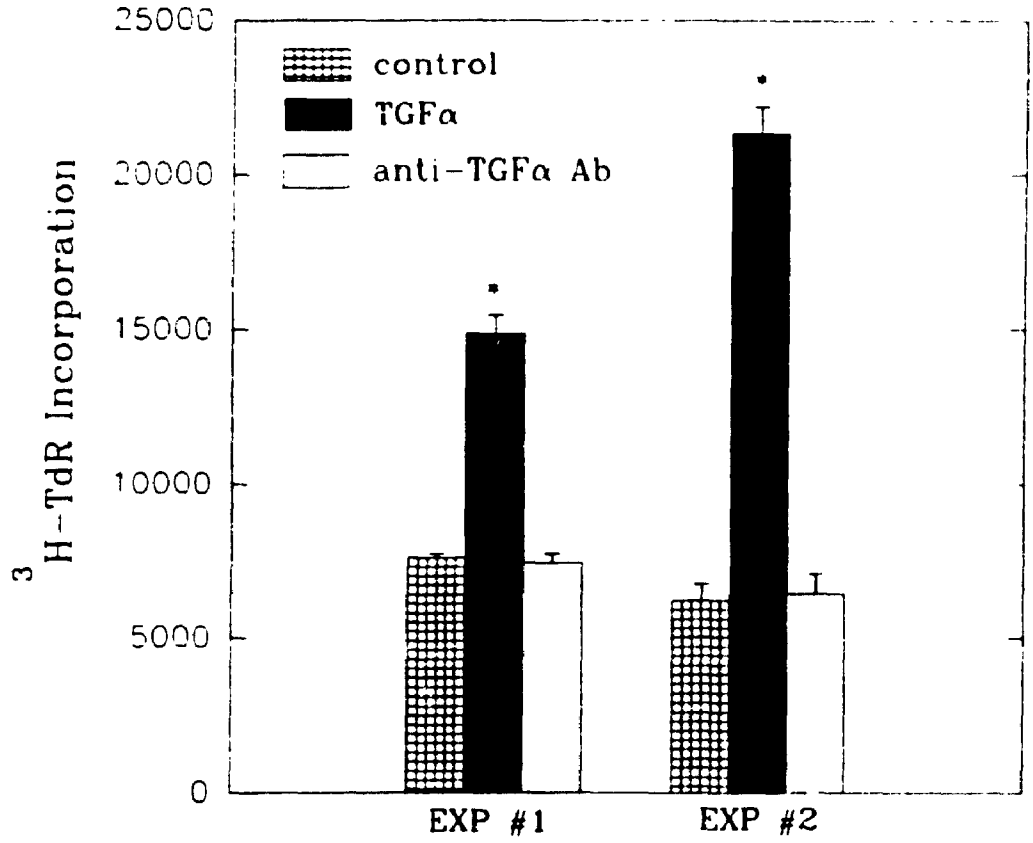

25000
20000
15000
10000
5000
0
control
TGFα
anti-TGFα Ab
^{3}H-TdR Incorporation
EXP #1
EXP #2

Figure 21: Effects of increasing doses (2.5, 5, 10, 20, and 40 μg/ml) of anti-TGF-α neutralizing Ab plus TGF-α (10 ng/ml) on ^{3}H-TdR incorporation by first trimester human trophoblast cells. Symbols represent the mean ($\pm$ SE) of quadruplicate wells. The SE of the data points for no additive and 20 and 40 μg/ml anti-TGFα Ab were too small to be plotted by the graphics program.

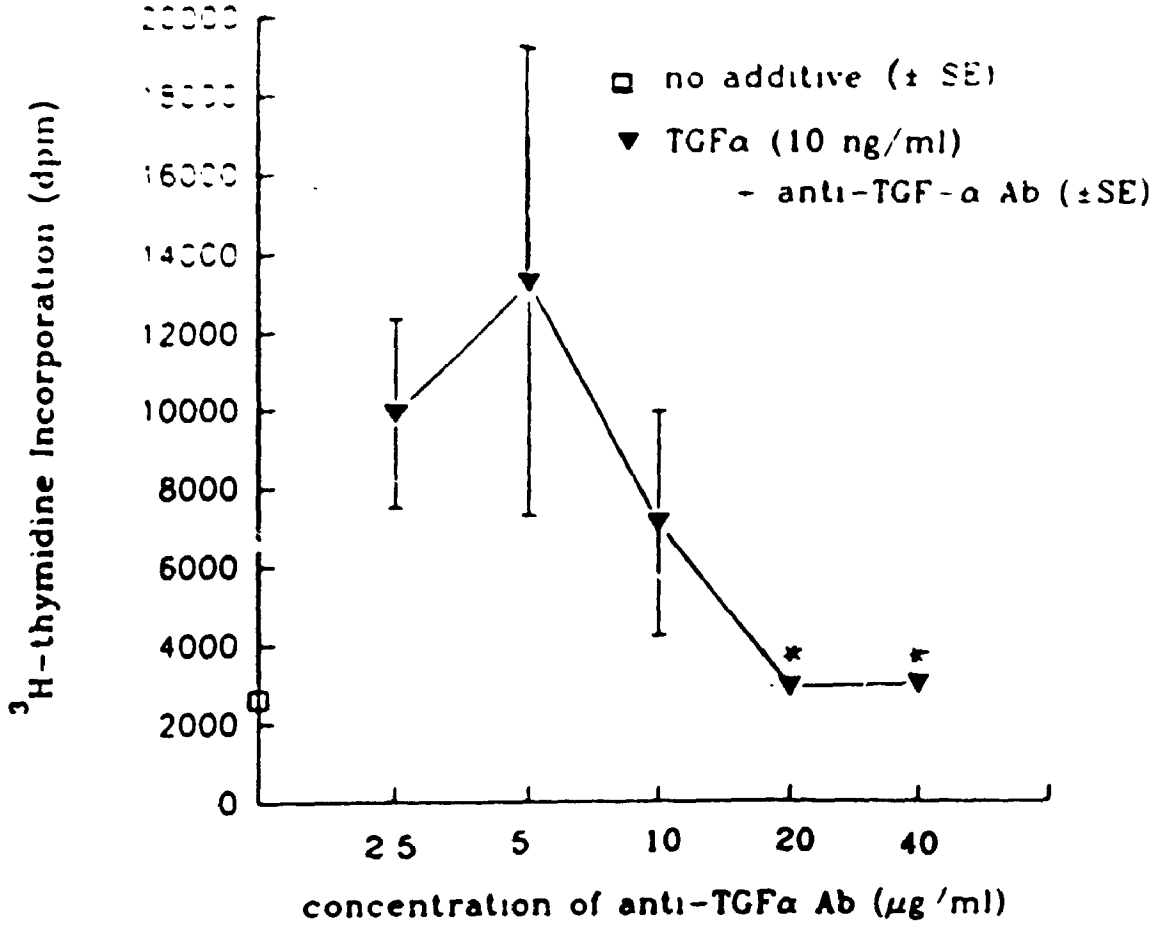

no additive (± SE)
TGFα (10 ng/ml)
+ anti-TGF-α Ab (±SE)
^{3}H-thymidine Incorporation (dpm)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
2.5
5
10
20
40
concentration of anti-TGFα Ab (μg/ml)

Figure 22: Effect of TGF-α (10 ng/ml), anti-TGF-α neutralizing Ab (25 μg/ml), and EGF (10 ng/ml) on ^{3}H-TdR incorporation by first trimester human trophoblast cells. Bars represent the mean ($\pm$ SE) of quadruplicate samples. * significantly different from the control value.

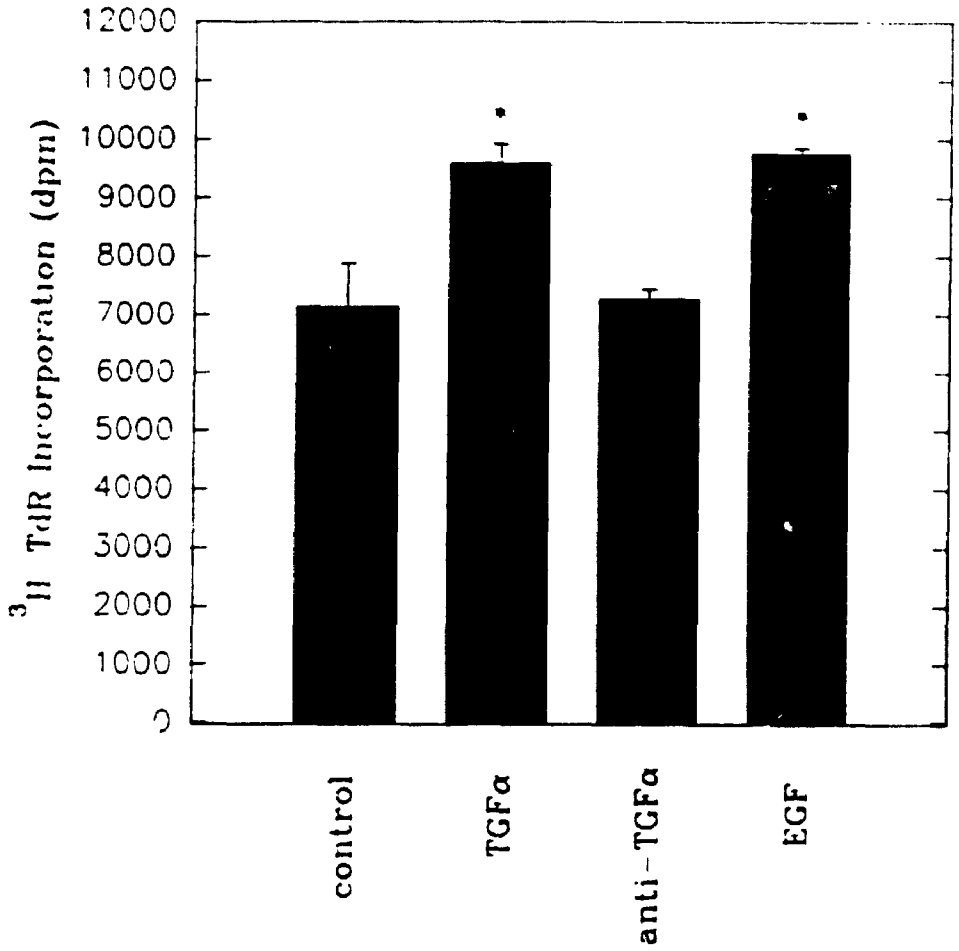
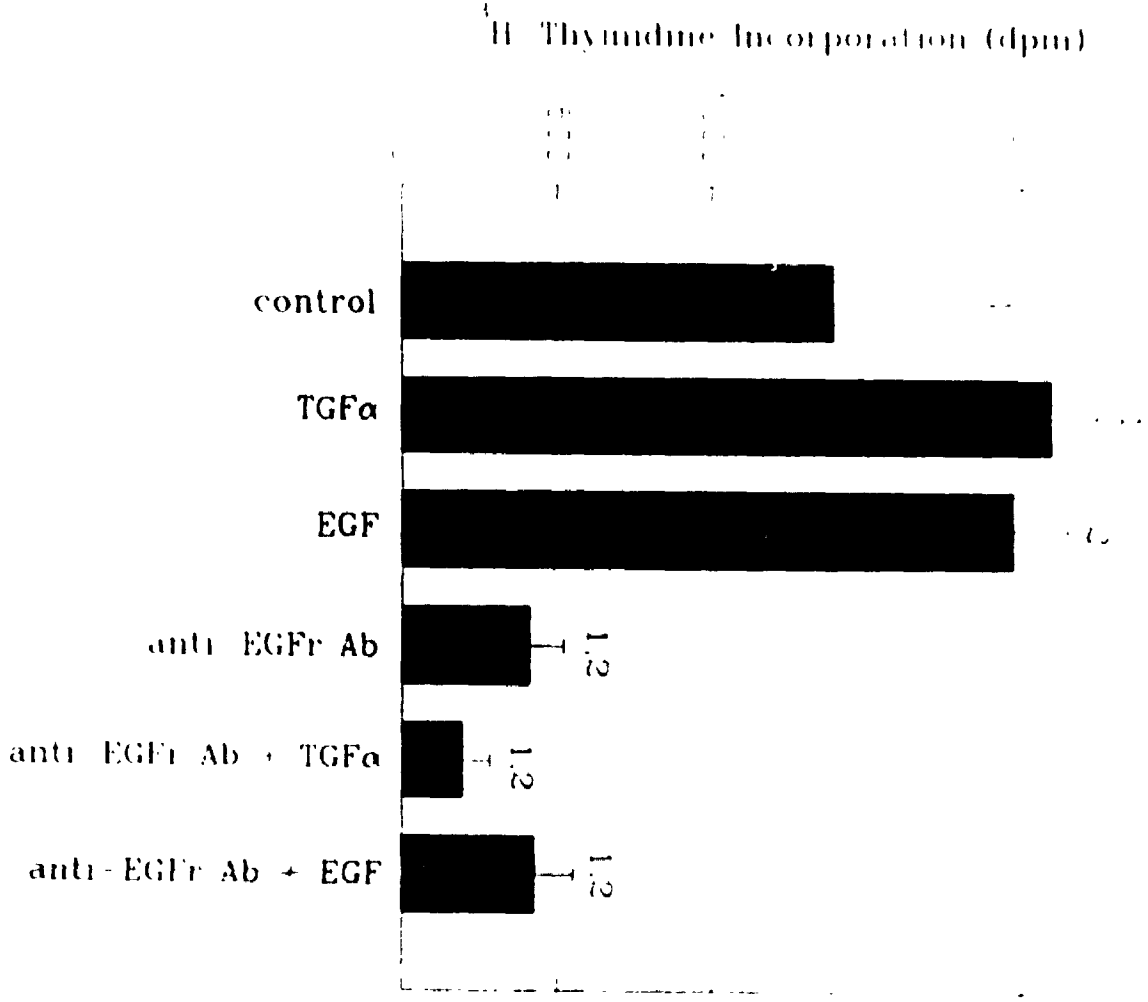

12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
^{3}H TdR Incorporation (dpm)
*
*
control
TGFα
anti-TGFα
EGF

Figure 23: Effect of TGF-α (10 ng/ml), EGF (10 ng/ml), anti-EGF-receptor blocking Ab (25 μg/ml), or a combination of TGF-α (10 ng/ml) or EGF (10 ng/ml) plus anti-EGF-receptor blocking Ab (25 μg/ml) on ^{3}H-TdR incorporation by first trimester human trophoblast cells. Bars represent the mean ($\pm$ SE) of quadruplicate wells. Numbers above the bars, in this and subsequent legends, indicate significant ($p \leq 0.05$) differences between bars with the same number.


control
TGFα
EGF
anti EGFr Ab
anti EGFr Ab + TGFα
anti-EGFr Ab + EGF

addition of anti-EGF-receptor blocking Ab alone caused a decrease in ^{3}H-TdR incorporation and this also blocked the proliferative stimulatory effects of EGF and TGFα at the above dose levels. These results indicate that endogenous growth stimulatory factor(s) which bind to the EGF-receptor have a significant role in proliferation in these trophoblast cultures.

Results from the PCNA immunostaining are shown in figure 24. As can be seen in figure 24 control cultures had very few nuclei immunoreactive for PCNA (0.7% $\pm$ .3) whereas cells treated in the presence of TGFα had almost a 5 fold increase in the mean percent labeled nuclei (3.3% $\pm$ 1.4), however this was not significant. Cultures incubated with EGF also showed an increase in the mean number of nuclei immunoreactive for PCNA (6.4% $\pm$ 5.5), which was not significant.

3.1.2. Effect of Amphiregulin on Trophoblast Cell Proliferation

The addition of increasing doses of AR to first trimester human trophoblast cells in culture caused a dose-dependent stimulation of proliferation (^{3}H-TdR uptake) witnin the dose range tested. A significant ($p < 0.01$) enhancement of proliferation was noted at 50 ng/ml AR. At 100 ng/ml AR the ^{3}H-TdR incorporation was 2.5 fold greater than the basal level. A plateau in ^{3}H-TdR incorporation was not achieved at the above doses (Figure 25).

3.2. The Effect of CSF-1 on Trophoblast Cell Proliferation

First trimester human trophoblast cells incubated with CSF-1 (10 ng/ml) showed no significant difference in their proliferation compared to control cultures (Figure 26).

Figure 24: Effect of TGF-α and EGF on PCNA immunostaining of early-passage first trimester human cells in vitro. Control, complete media only; TGF-α, 10 ng/ml TGF-α; EGF, 10 ng/ml EGF. Bars represent the mean ($\pm$ SE) of triplicate samples.

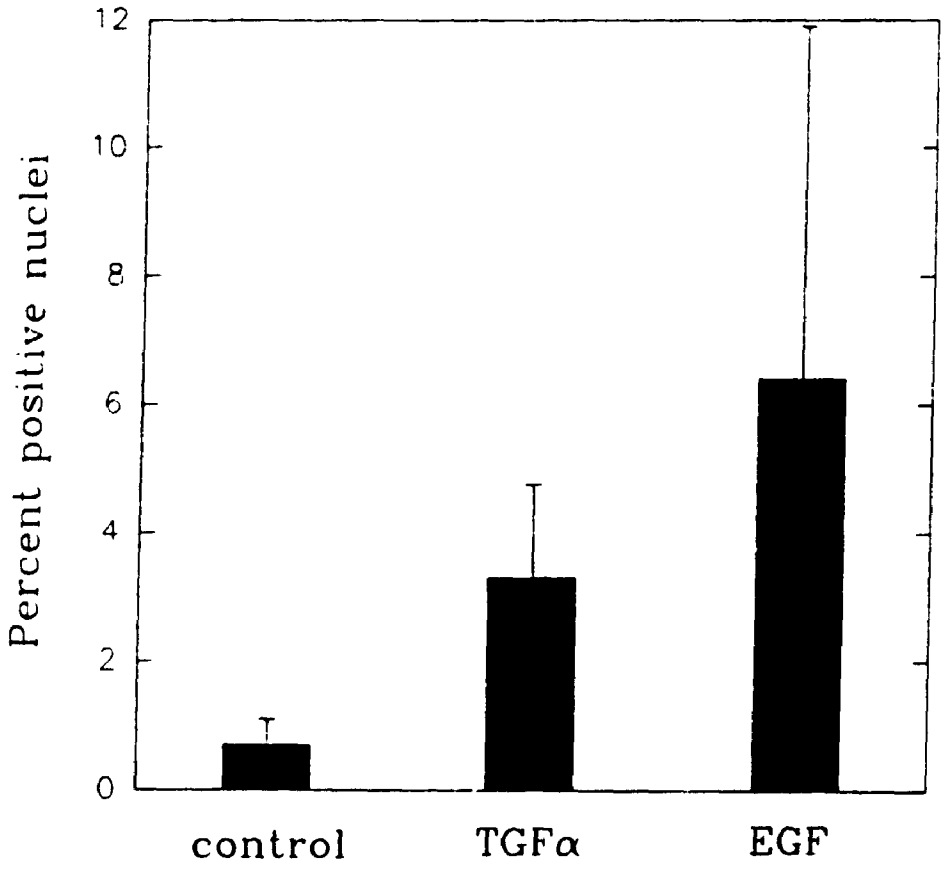

Percent positive nuclei
12
10
8
6
4
2
0
control
TGFα
EGF

Figure 25: Effect of various concentrations (0 - 100 ng/ml) of AR on ^{3}H-TdR incorporation by first trimester human trophoblast cells grown in a serum free media (ExCell, JRH Biosciences). Symbols represent the mean ($\pm$ SE) of quadruplicate wells. * significantly different from the value at 0 ng/ml.

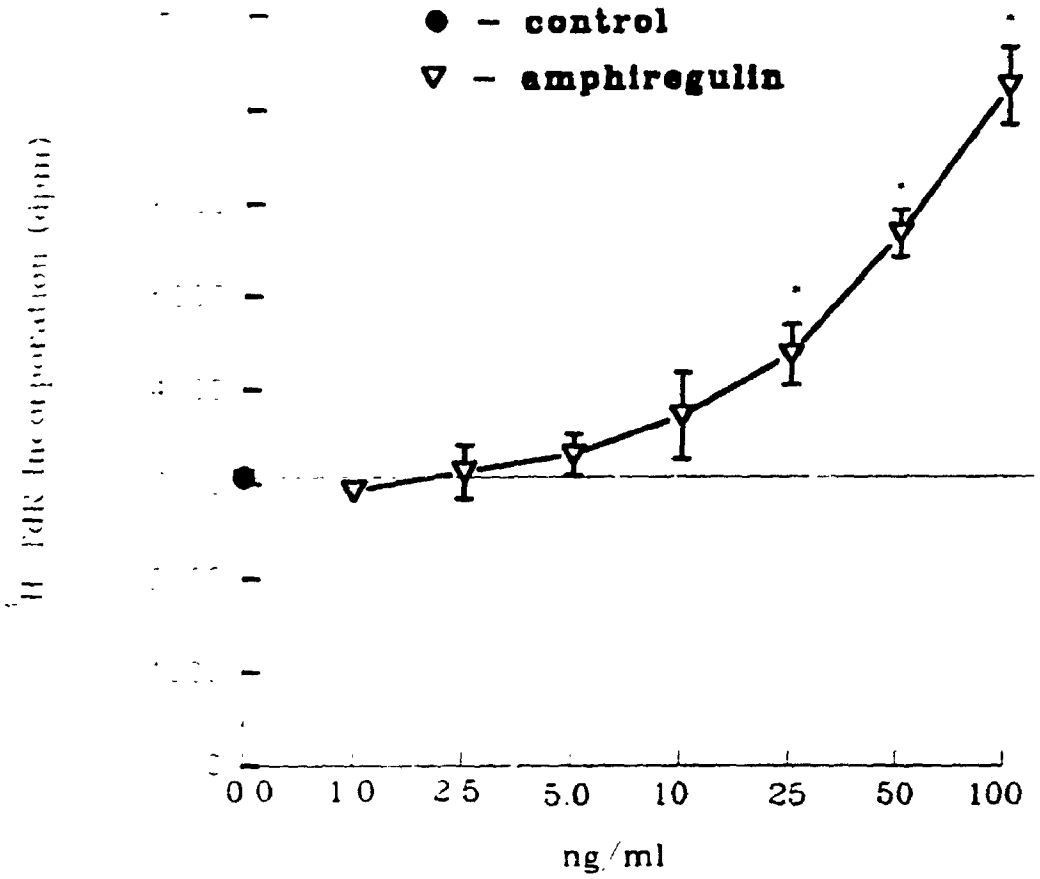

● – control
▽ – amphiregulin
0 0
1 0
2 5
5.0
10
25
50
100
ng/ml

Figure 26: Effect of CSF-1 on ^{3}H-TdR incorporation by first trimester human trophoblast cells. Bars represent the mean ($\pm$ SE) of quadruplicate samples. Control, complete media only; CSF-1, 10 ng/ml CSF-1.

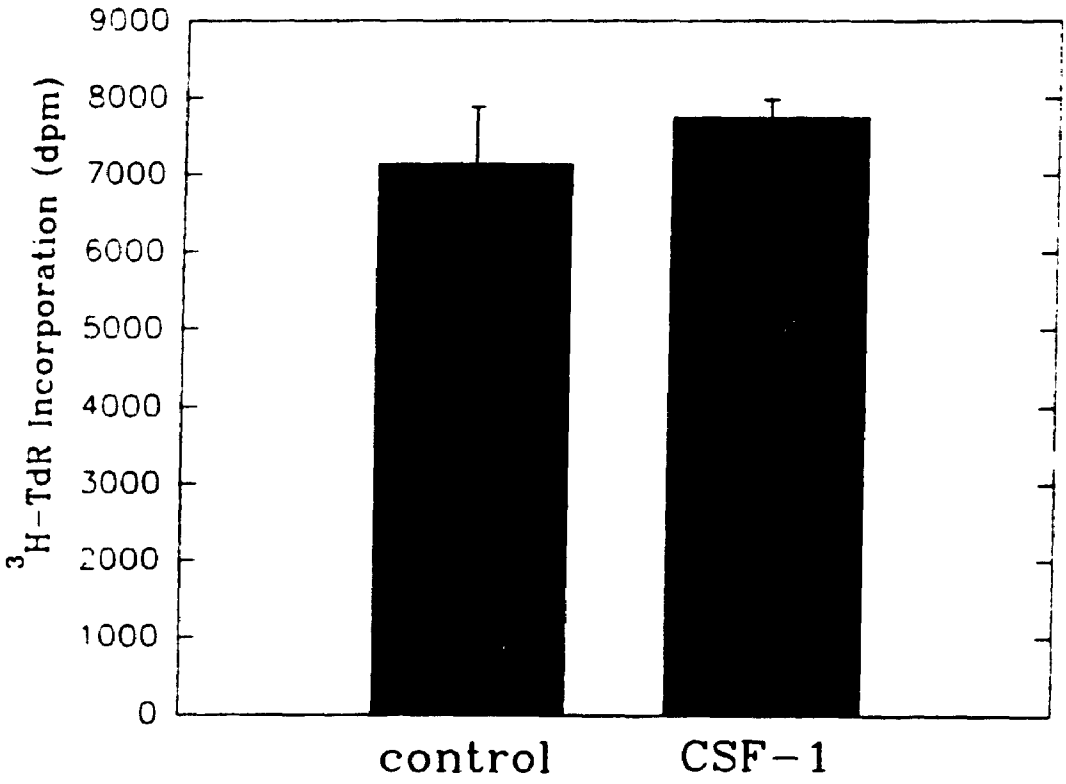
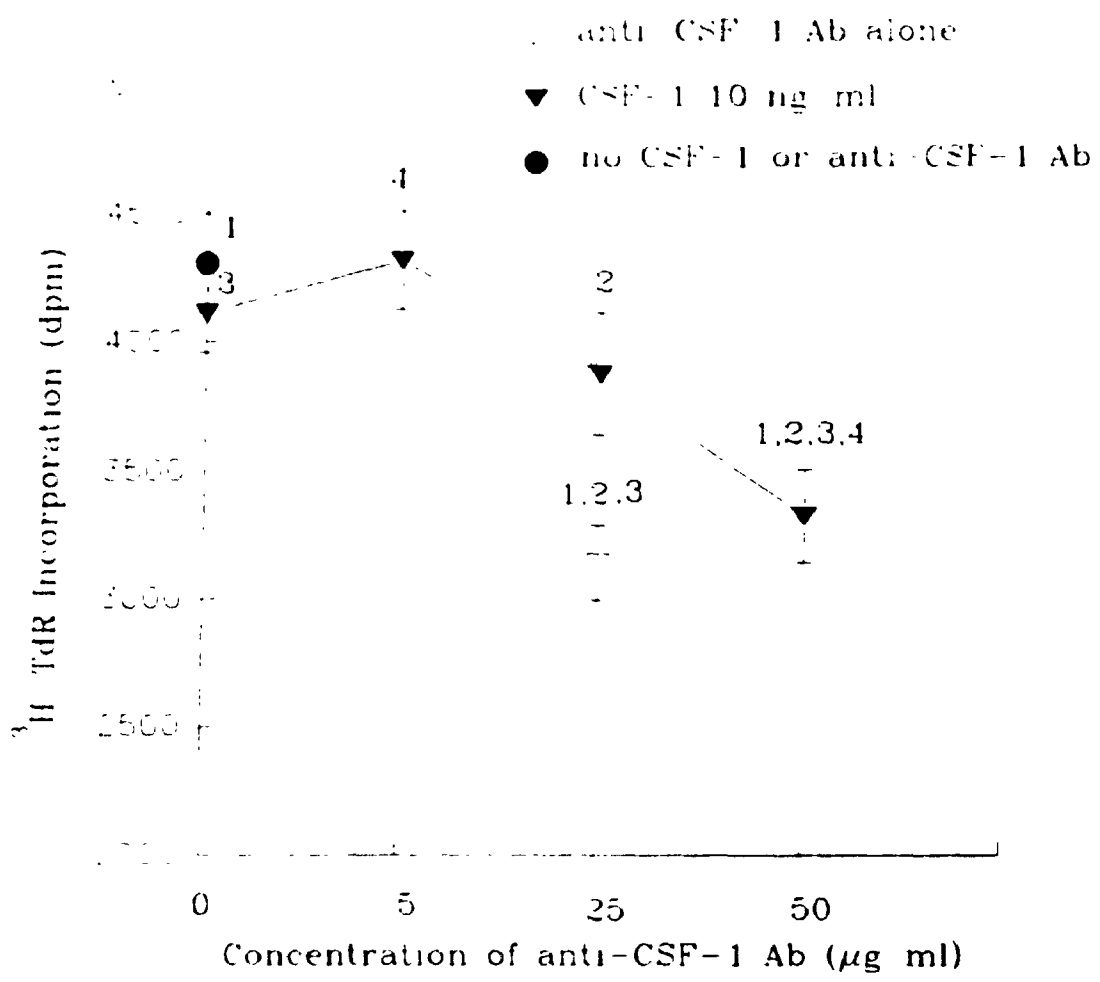

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
^{3}H-TdR Incorporation (dpm)
control
CSF-1

Figure 27: The effect of increasing doses (0 - 50 μg/ml) of anti-CSF-1 neutralizing Ab in combination with CSF-1 (10 ng/ml), anti-CSF-1 neutralizing Ab (25 μg/ml) alone, and no additive on the ^{3}H-TdR incorporation by first trimester human trophoblast cells. Symbols represent the meas ($\pm$ SE) of triplicate wells. Corresponding numbers (1,2,3,4) appearing on data points indicate a significant difference.


anti CSF-1 Ab alone
CSF-1 10 ng/ml
no CSF-1 or anti-CSF-1 Ab
^{3}H TdR Incorporation (dpm)
1
3
4
2
1,2,3
1,2,3,4
2500
0
5
25
50
Concentration of anti-CSF-1 Ab (μg/ml)

However, trophoblast cells incubated in the presence of anti-CSF-1 neutralizing Ab (25 μg/ml) showed a significant ($p < 0.01$) decrease in proliferation compared to control cultures (Figure 27). This effect was partially abrogated by the addition of 10 ng/ml CSF-1 to the Ab treated cultures (Figure 27). These results indicate the presence of endogenous, growth stimulatory, CSF-1 in these cultures.

3.3. The Effect of IGF-II on Trophoblast Cell Proliferation

The addition of increasing doses of IGF-II (0 - 100 ng/ml) had no significant influence on first trimester human trophoblast cell proliferation (Figure 28).

4. THE EFFECT OF TGFα ON MULTINUCLEATE CELL FORMATION BY TROPHOBLAST

Incubation of first trimester trophoblast cells with TGFα (10 ng/ml) for 72h resulted in a shift in the ratio of uninucleate cell to multinucleate cells. Figure 29 shows that exogenously added TGFα to the culture increased the incidence of uninucleate cells (56.9% $\pm$ 3.28) compared to control cultures (38% $\pm$ 1.7) ($p < 0.05$). Addition of the TGFα neutralizing Ab alone did not alter the ratio of uninucleate to multinucleate cells.

5. THE EFFECTS OF TGFα, EGF, CSF-1, AND IGF-II ON TROPHOBLAST INVASIVENESS IN VITRO

Results of a 3 day matrigel invasion assay with first trimester human trophoblast

Figure 28: The effect of increasing doses (0 - 100 ng/ml) of IGF-II on first trimester human trophoblast cell proliferation. This experiment was performed in the presence of a serum free media (ExCell). Symbols represent the mean (± SE) of quadruplicate samples.

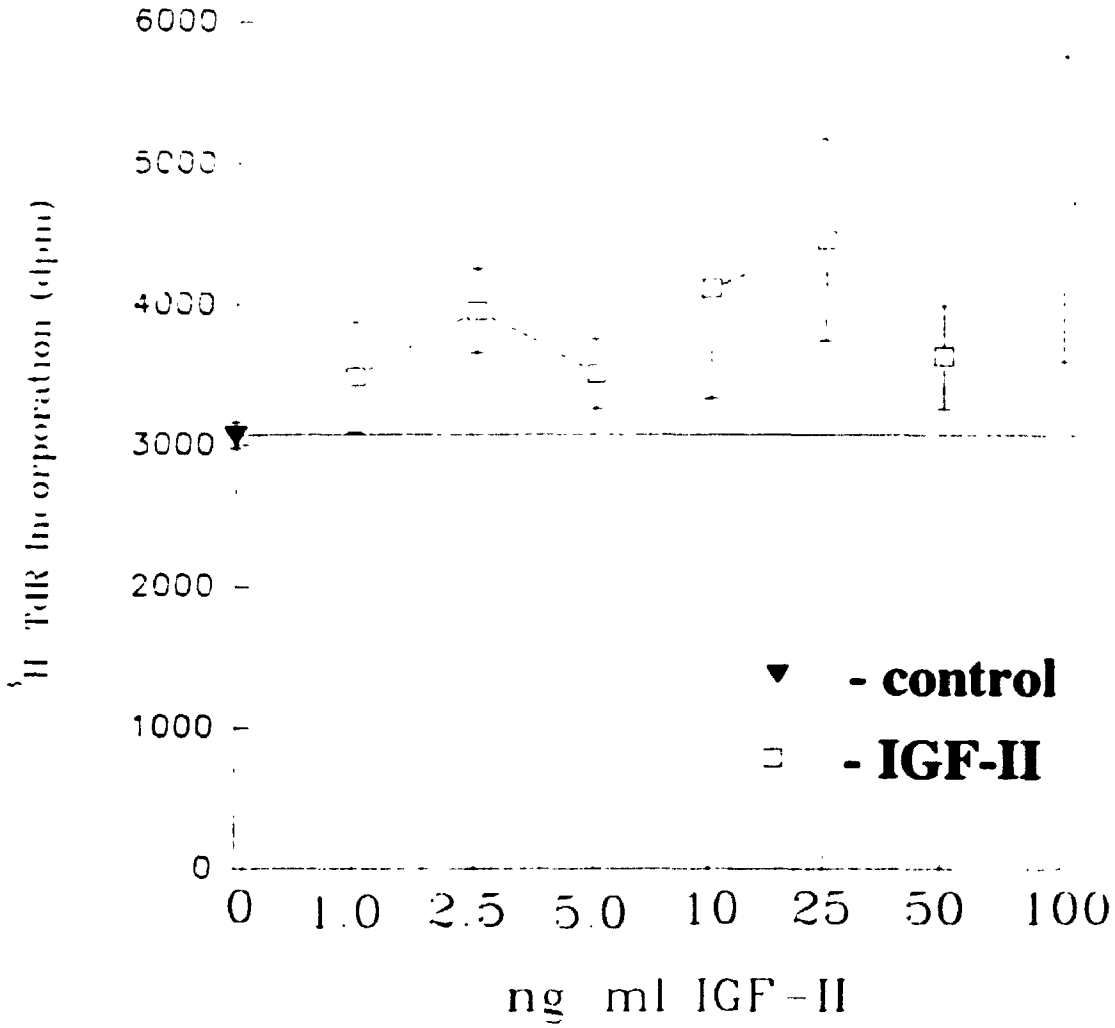

6000
5000
4000
3000
2000
1000
0
^{3}H TdR Incorporation (dpm)
▼ - control
□ - IGF-II
0 1.0 2.5 5.0 10 25 50 100
ng ml IGF-II

Figure 29: Effect of TGF-α on the formation of multinucleate cells by first trimester human trophoblast cells. A total of 5000 cells were counted in quadruplicate wells under each condition. Multinucleated cells were scored as cells containing 2 or more nuclei. Bars represent the mean ($\pm$ SE) percentage of the total number of cells counted. Control, complete media only; TGF-α, 10 ng/ml TGF-α; anti-TGF-α, 25 μg/ml Ab. Corresponding numbers (1,2) appearing on bars indicate a significant difference between the pair of values.

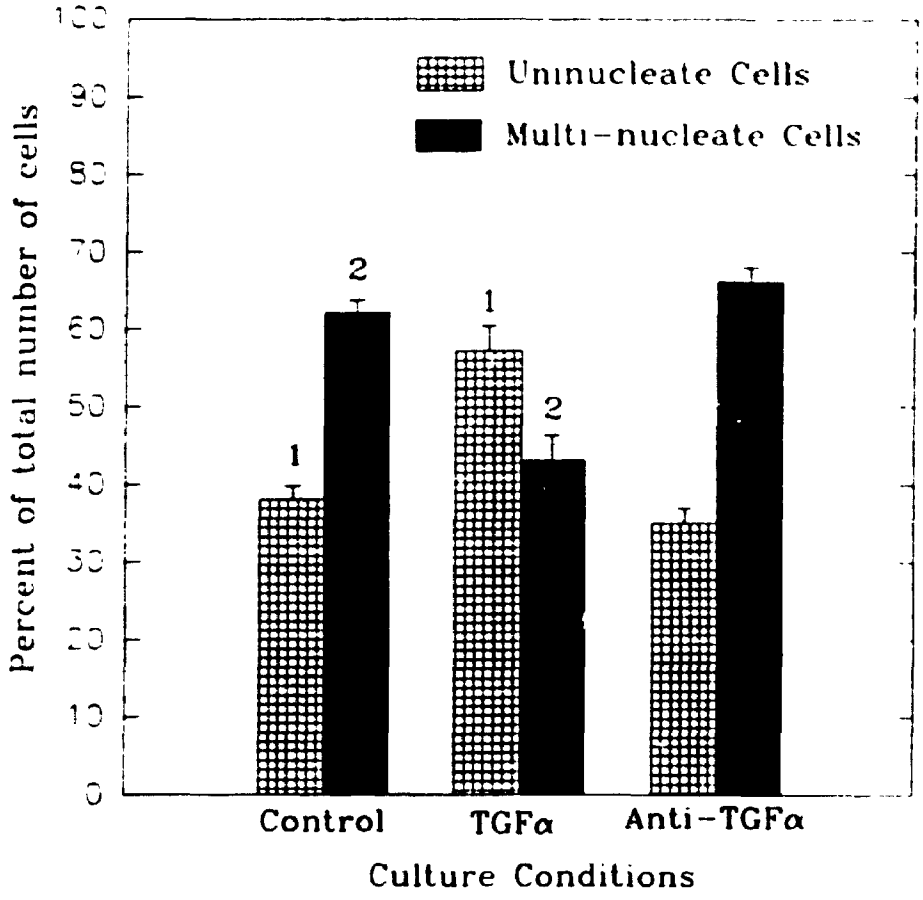
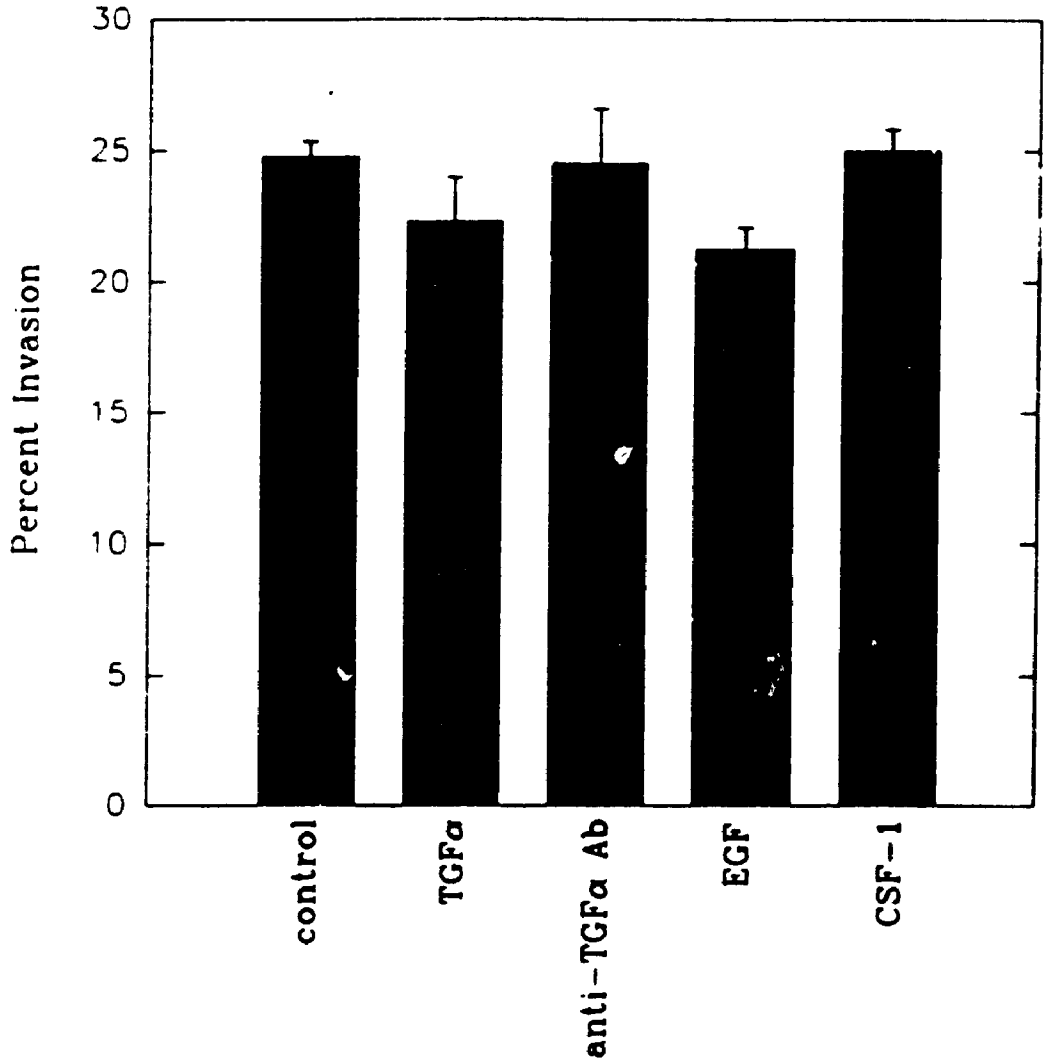

Uninucleate Cells
Multi-nucleate Cells
Percent of total number of cells
100
90
80
70
60
50
40
30
20
10
0
1
2
1
2
Control
TGFα
Anti-TGFα
Culture Conditions

Figure 30: Results of a 3 day matrigel invasion assay with early passage first trimester human trophoblast cells treated with TGF-α, EGF, CSF-1 (all at a concentration of 10 ng/ml), or anti-TGF-α neutralizing Ab (25 μg/ml). None of the treatments caused significant changes in the invasiveness of the first trimester trophoblast cells. Bars represent the mean ($\pm$ SE) of triplicate wells.


Percent Invasion
30
25
20
15
10
5
0
control
TGFα
anti-TGFα Ab
EGF
CSF-1

cells treated with EGF (10 ng/ml), TGFα (10 ng/ml), anti-TGFα neutralizing Ab (25 μg/ml), CSF-1 (10 ng/ml), or no additive are presented in Figure 30. Doses of the growth factors used in the invasion assays where concentrations which demonstrated proliferative responses. None of the treatments altered the invasion index of trophoblast cells as compared to untreated cells.

Figure 31 shows the results of a 3 day matrigel invasion assay with increasing doses of IGF-II performed in serum free (Excell) media. As can be seen in this figure, the control (no additive) level of invasion in serum free Excell media is more than triple the level of the control value when the invasion assay was performed with complete medium (RPMI 1640 plus 10% FCS). Increasing concentrations of IGF-II in Excell media caused a slight nonsignificant stimulation of the already elevated levels of invasion. Enquiries with the supplier (JRH Biosciences, Lenexa, KS) revealed that the Excell medium contains very high levels of insulin (5 μg/ml) in addition to transferrin, selenium, and other undisclosed molecules. It was suspected that high insulin levels may have been responsible for the very high basal level of invasion measured in Excell medium.

Results of matrigel invasion assays with increasing doses of IGF-II and in the presence of different serum concentrations in RPMI 1640 medium (GIBCO) are presented in figures 32 and 33. When the experiment was performed in media containing 10% serum, increasing concentrations of IGF-II had no significant effect on trophoblast invasion; however, when the serum concentration was reduced to 1%, trophoblast invasion was enhanced with IGF-II in a concentration-dependent manner (Figure 32). In figure 33, a concentration-dependent increase in invasion is again noted with IGF-II

Figure 31: Results of a 3 day matrigel invasion assay with early passage first trimester human trophoblast cells performed in a serum free media (ExCell). As can be seen there is an approximately 3 fold increase in trophoblast cell invasiveness in cell cultured in the serum free media (ExCell; control, serum free) compared to cells cultured in complete media (control, complete media, RPMI 1640 plus 10% FCS). Both of these treatments were done at exactly the same time from the same fla. . of radiolabeled cells. Cells treated with increasing doses of IGF-II (0 - 500 ng/ml) showed a slight, nonsignificant stimulation of invasion, over the already elevated levels. Bars represent the mean ($\pm$ SE) of triplicate wells.

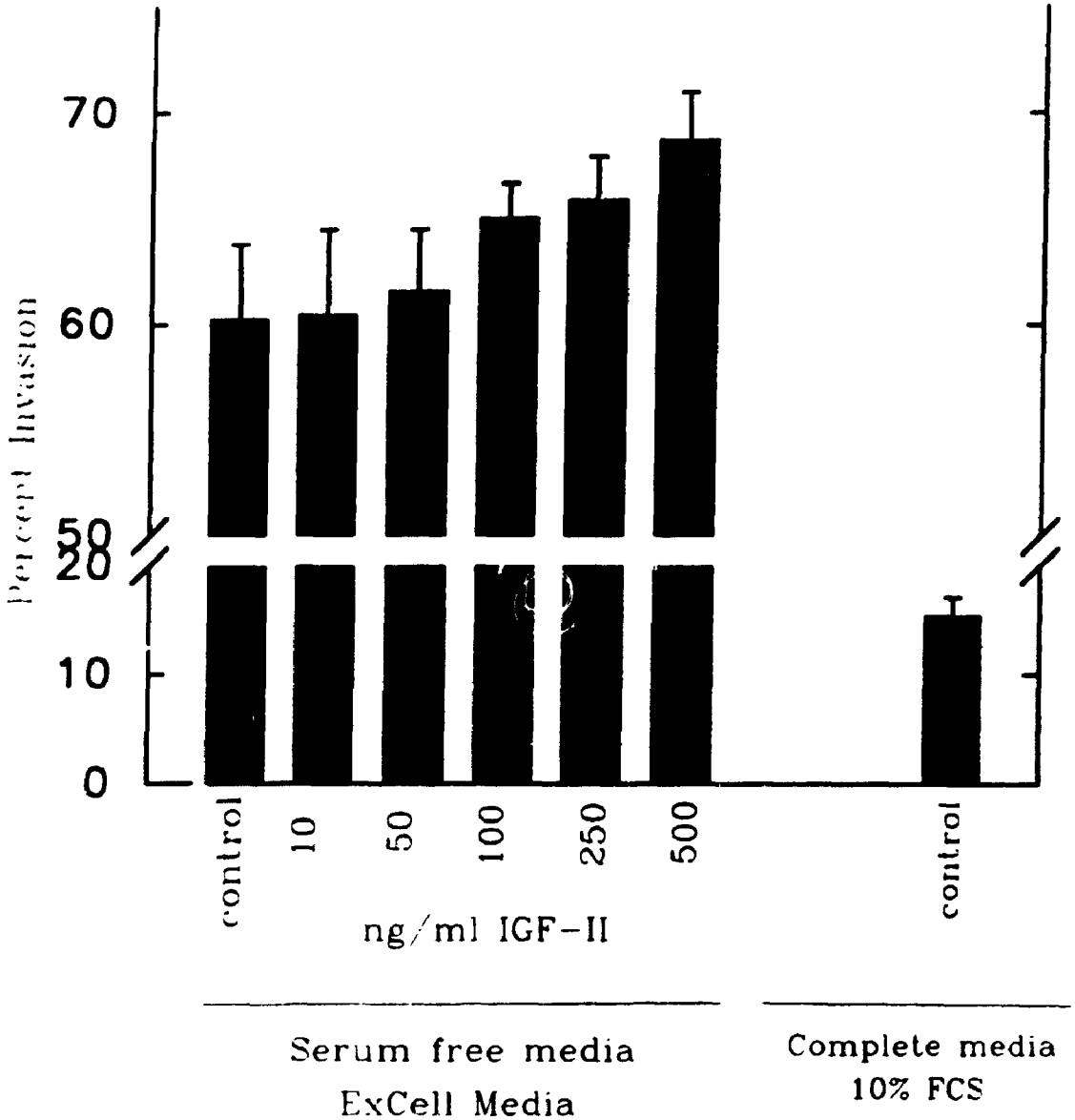

Percent Invasion
70
60
50
28
10
0
control
10
50
100
250
500
control
ng/ml IGF-II
Serum free media
ExCell Media
Complete media
10% FCS

Figure 32: Results of a 3 day matrigel invasion assay with first trimester trophoblast cells treated with increasing concentrations of IGF-II (0 - 600 ng/ml) under two separate conditions, 1) cultured during the assay in complete media (10% FCS) and 2) cultured during the assay in serum reduced media (1% FCS). No response to the IGF-II, at any dose is seen when the assay was performed in the presence of 10% FCS; however, in the presence of 1% FCS a dose response increase in trophoblast invasiveness is seen. Bars represent the mean ($\pm$ SE) of triplicate wells. * indicates significantly different from control values.

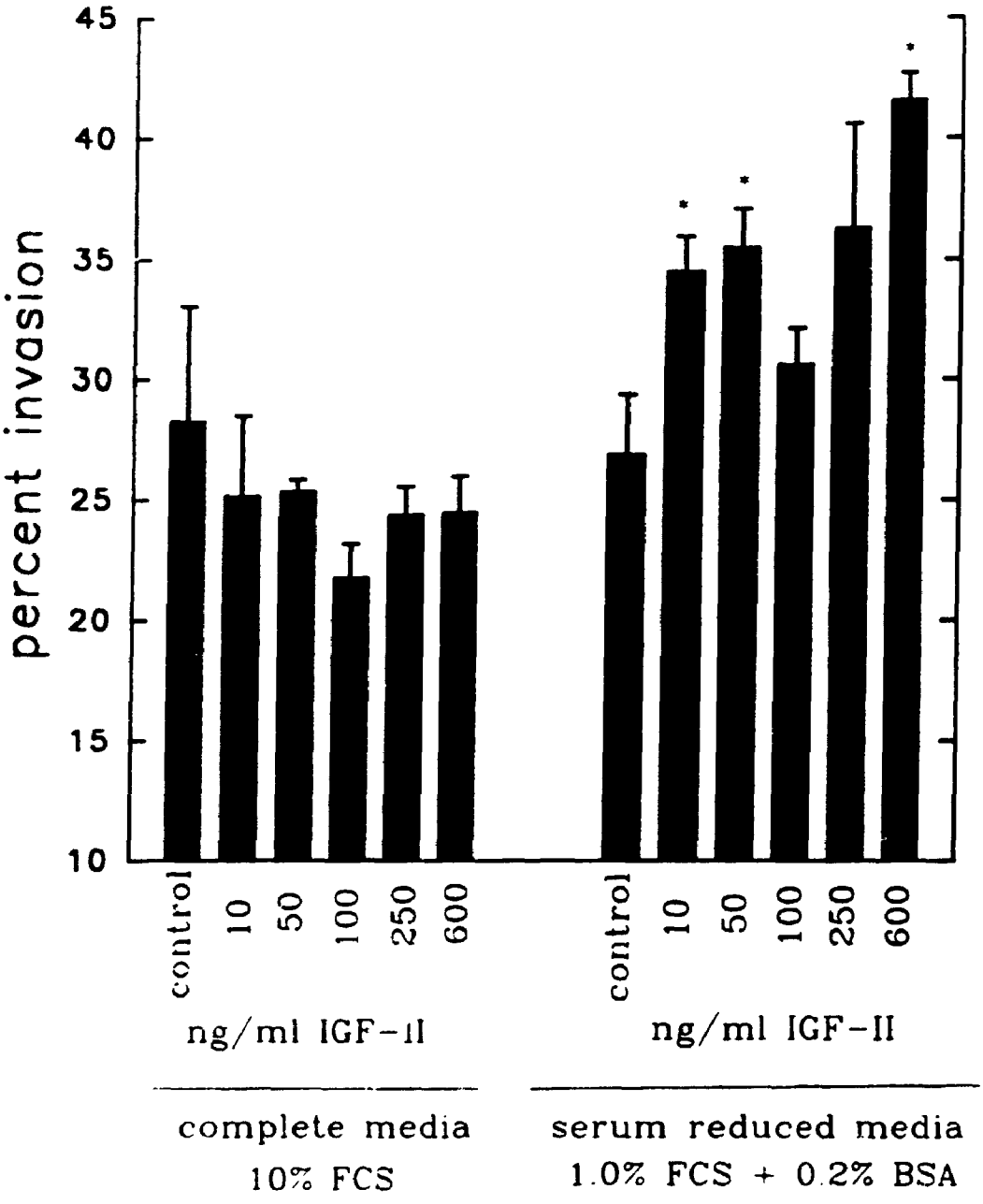
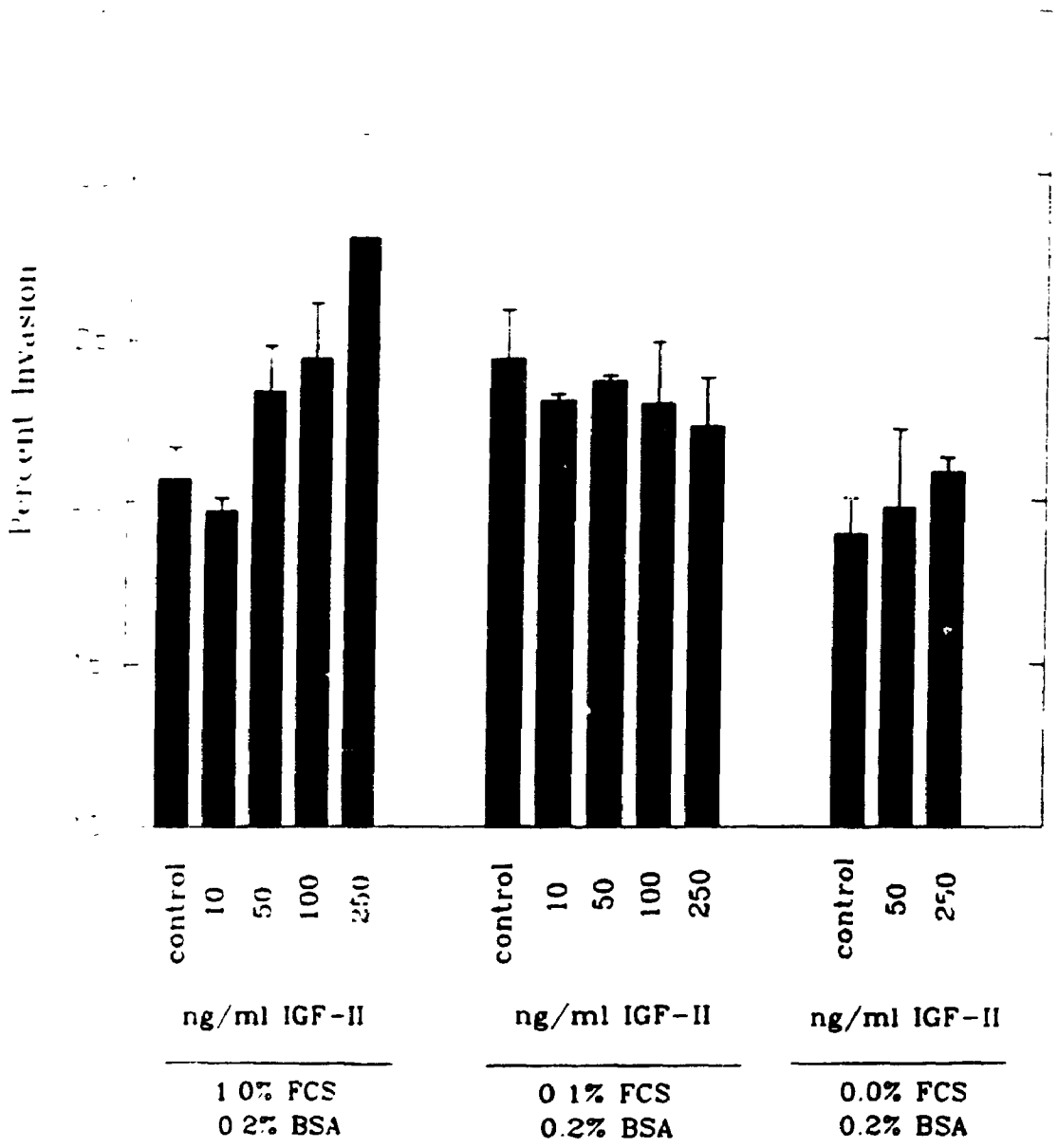

45
40
35
30
25
20
15
10
percent invasion
control
10
50
100
250
600
ng/ml IGF-II
control
10
50
100
250
600
ng/ml IGF-II
complete media
10% FCS
serum reduced media
1.0% FCS + 0.2% BSA

Figure 33: Results of a 3 day matrigel invasion assay with first trimester trophoblast cells treated with increasing doses of IGF-II (0 - 250 ng/ml) under three separate conditions, 1) cultured in the presence of 1% FCS, 2) cultured in the presence of 0.1% FCS, and 3) cultured in the presence of 0% FCS. In the presence of 1% FCS a stimulation of trophoblast invasiveness is seen again (as in figure 32). Invasion assays performed in the presence of 0.1% and 0% FCS displayed no significant response to increasing doses of IGF-II. Bars represent the mean ($\pm$ SE) of triplicate wells.

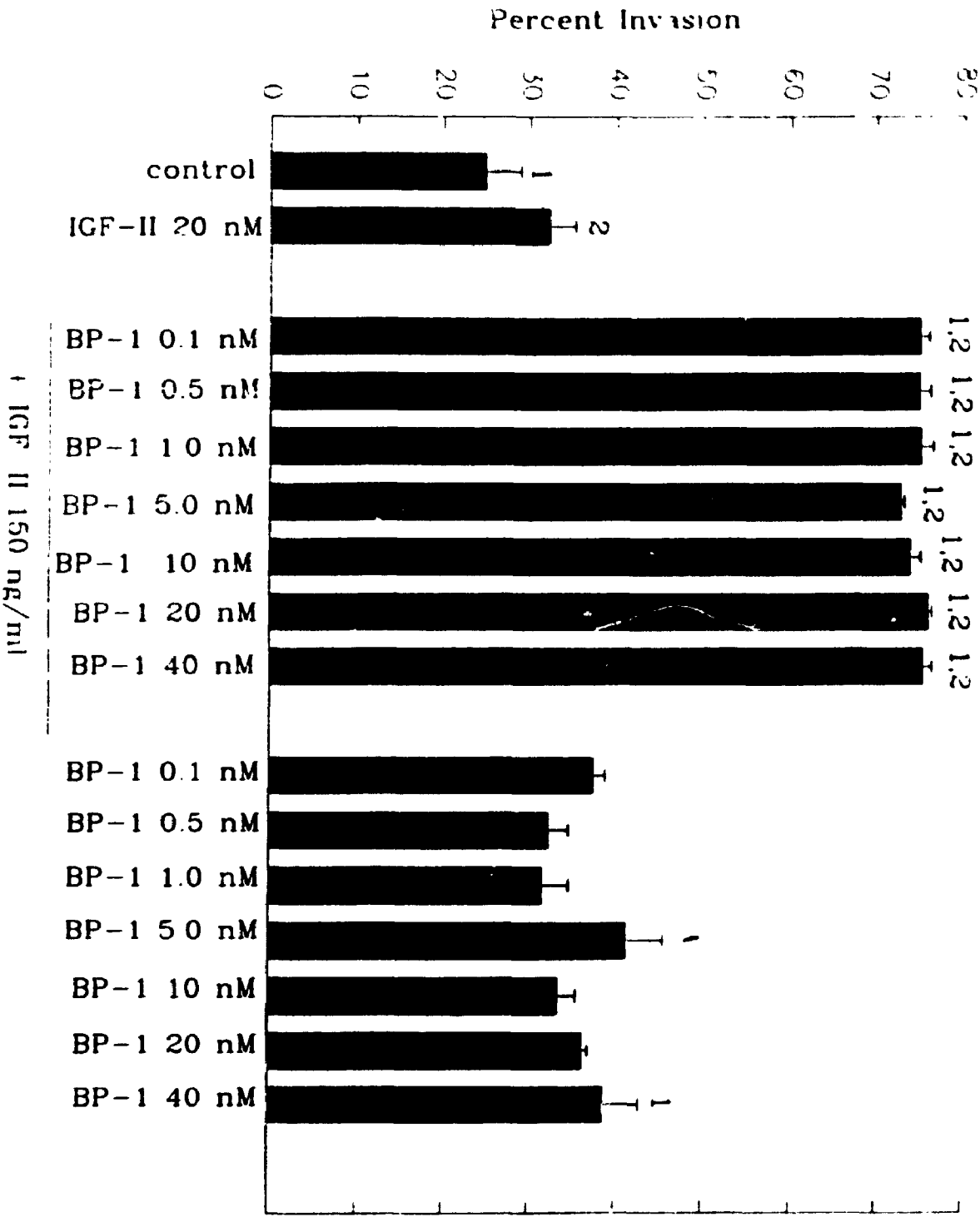

Percent Invasion
control
10
50
100
250
ng/ml IGF-II
1.0% FCS
0.2% BSA
control
10
50
100
250
ng/ml IGF-II
0.1% FCS
0.2% BSA
control
50
250
ng/ml IGF-II
0.0% FCS
0.2% BSA

in media containing 1% serum. Further reduction in serum concentration (0.1% and 0% serum) abolished the invasion stimulatory effects of IGF-II. These results suggest that FCS contains molecule(s) required for IGF-II stimulatory effects, and also inhibitory molecule(s) at high serum concentration (10%) that may counteract IGF-II stimulation. IGF binding proteins present in the serum may have been responsible for the results.

Results of immunolocalization (of protein) and in situ hybridization (of mRNA) of IGF-II and its binding protein IGFBP-1 by Dr. Victor Han (Lawson Research Institute; Han, V. unpublished data) in the human placenta and the decidua throughout gestation strongly suggests an interaction between IGF-II produced by the invasive intermediate trophoblast and IGFBP-1 produced by decidual cells in the vicinity of the invasive trophoblast in situ. As shown in figure 4 (courtesy of Dr. V. Han), both IGF-II and IGFBP-1 proteins were present in the trophoblast as well as decidual cells. However, IGF-II mRNA was selectively localized to the extravillous invasive trophoblast cells throughout gestation; whereas, IGFBP-1 mRNA was selectively localized to the decidual cells.

To investigate the possible interaction of IGF-II with its binding protein, IGFBP-1, during trophoblast invasion, invasion assays were performed in the presence of IGF-II and different concentration of IGFBP-1 or IGFBP-1 alone in serum reduced (1% FCS) media. As evident in figure 34, increasing doses of IGFBP-1 (0.1 - 40 nM) alone enhanced trophoblast invasiveness compared to cells treated with no additive. IGF-II alone (20 nM or 150 ng/ml) caused an increase in invasion. However, all concentrations of IGFBP-1 in combination with a fixed dose of IGF-II greatly stimulated trophoblast invasion over cells treated with IGF-II alone, IGFBP-1 alone, or with no additive. The

Figure 34: Results of 3 day matrigel invasion assay with first trimester trophoblast cells treated with: 1) IGF-II, 20 nM (150 ng/ml); 2) increasing doses of IGFBP-1, 0.1 - 40 nM; 3) increasing doses of IGFBP-1, 0.1 - 40 nM plus IGF-II, 20 nM (150 ng/ml); and control, no additive. Cells treated with 20 nM IGF-II showed enhanced invasiveness over control cultures. In the presence of IGFBP-1 a stimulation of invasion was also seen over control cultures. A combination of IGF-II and all doses of IGFBP-1 caused a significant stimulation of invasion compared to control ($p < 0.01$), cultures treated with IGF-II (20 nM) alone ($p < 0.01$), or IGFBP-1 alone. The combined effects were more than additive, indicating synergy. Bars represent the mean ($\pm$ SE) of triplicate wells. Corresponding numbers (1,2) appearing on bars indicate a significant difference.


Percent Invasion
0
10
20
50
40
50
60
70
control
IGF-II 20 nM
BP-1 0.1 nM
BP-1 0.5 nM
BP-1 1 0 nM
BP-1 5.0 nM
BP-1 10 nM
BP-1 20 nM
BP-1 40 nM
1.2 1.2 1.2 1.2 1.2 1.2 1.2
+ IGF II 150 ng/ml
BP-1 0.1 nM
BP-1 0.5 nM
BP-1 1.0 nM
BP-1 5 0 nM
BP-1 10 nM
BP-1 20 nM
BP-1 40 nM

effects were synergistic in nature.

6. THE EFFECTS OF EGF, TGFα AND CSF-1 ON STEADY-STATE LEVELS OF TROPHOBLAST INVASION-REGULATING MOLECULES

Northern blot analysis of the invasion-regulating molecules TIMP-1, TIMP-2, and 72 and 92 kDa type IV collagenase was performed on total RNA from trophoblast cells treated with either EGF (10 ng/ml), TGFα (10 ng/ml), anti-TGFα neutralizing Ab (25 μg/ml), CSF-1 (10 ng/ml) or no additive. mRNA expression for the 72 kDa type IV collagenase showed a slight increase in cells treated with EGF and no appreciable change in cells treated with TGFα or anti-TGFα neutralizing Ab (Figure 35a). EGF and TGFα upregulated TIMP-1 mRNA expression as compared to cells treated with anti-TGFα neutralizing Ab or no additive (Figure 35b). Northern blot analysis of TIMP-2 mRNA revealed a small increase in the 3.5 kb message in cells cultured with EGF and TGFα (Figure 35c).

Trophoblast cells treated with CSF-1 showed a slight upregulation in the mRNA levels of the 72 kDa type IV collagenase and TIMP-1; whereas, TIMP-2 mRNA levels remained unchanged (Figure 36a-c).

The 92 kDa type IV collagenase mRNA was not detected by Northern analysis of total RNA isolated from cells incubated under control or experimental conditions (data not shown).

Figure 35: Northern blot analysis to examine the effects of TGF-α, EGF, and anti-TGF-α neutralizing Ab on the mRNA levels of the invasion regulating molecules, 72 kDa type IV collagenase (3.2 kb), TIMP-1 (1.0 kb), and TIMP-2 (3.5 and 0.9 kb).

a) mRNA levels of the 72 kDa type IV collagenase enzyme were slightly upregulated in cells cultured in the presence of EGF (10 ng/ml)but not with TGF-α (10 ng/ml) as compared to the untreated control. Treatment with anti-TGF-α Ab (25 μg/ml) did not have any appreciable effect.

b) TIMP-1 mRNA levels were upregulated in trophoblast cells treated with TGF-α and EGF, compared to control and anti-TGF-α Ab treated cultures, which exhibited similar levels of message.

c) TIMP-2 mRNA (3.5 kb message) levels were also slightly upregulated in cells treated with TGF-α and EGF, compared to control cells.

FIRST TRIMESTER HUMAN TROPHOBLAST CELLS

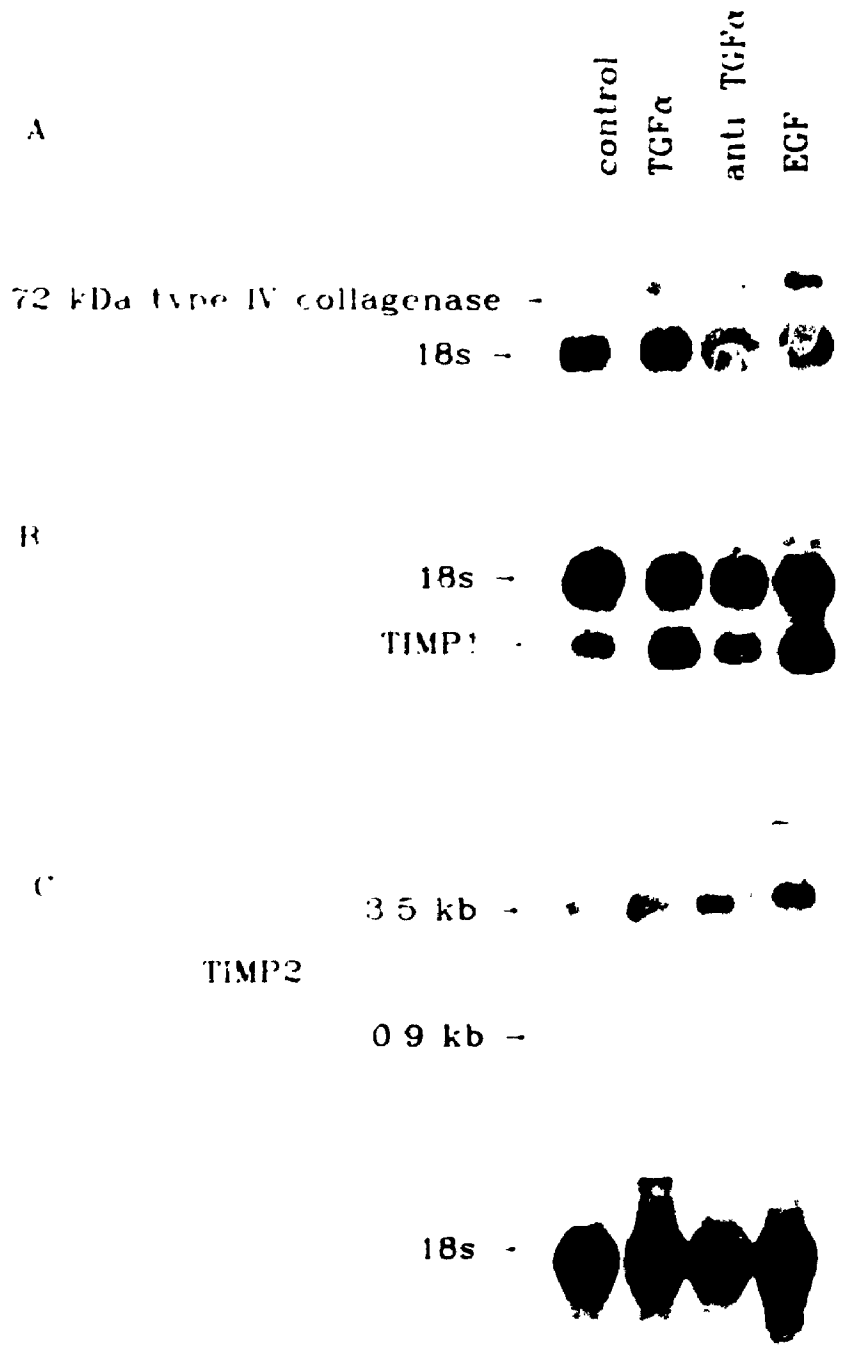


Figure 36: Northern blot analysis to examine the effects of CSF-1 on the mRNA levels of the invasion regulating molecules, 72 kDa type IV collagenase, TIMP-1, and TIMP-2.

a) mRNA levels of the 72 kDa type IV collagenase enzyme were stimulated in cultures treated with CSF-1 (10 ng/ml) compared to control cultures.

b) TIMP-1 mRNA levels were also slightly upregulated in cultures treated with CSF-1 compared to control cultures.

c) TIMP-2 mRNA levels remained relatively unchanged in CSF-1 treated cells.

FIRST TRIMESTER HUMAN TROPHOBLAST CELLS

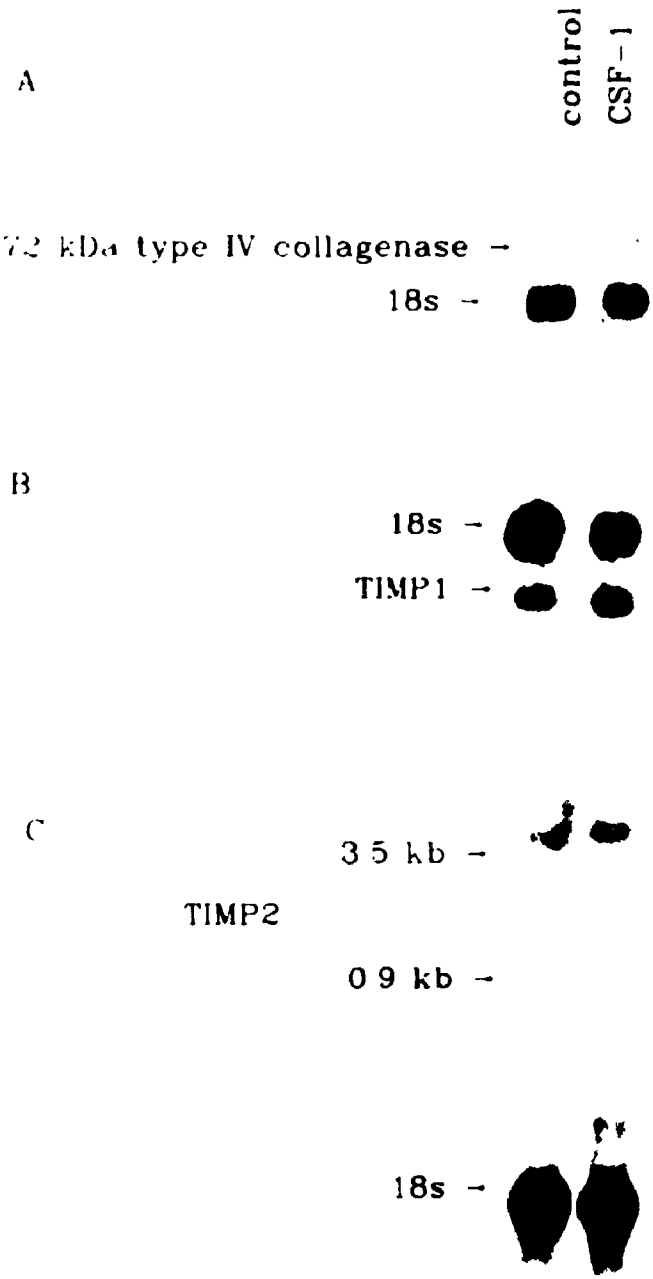
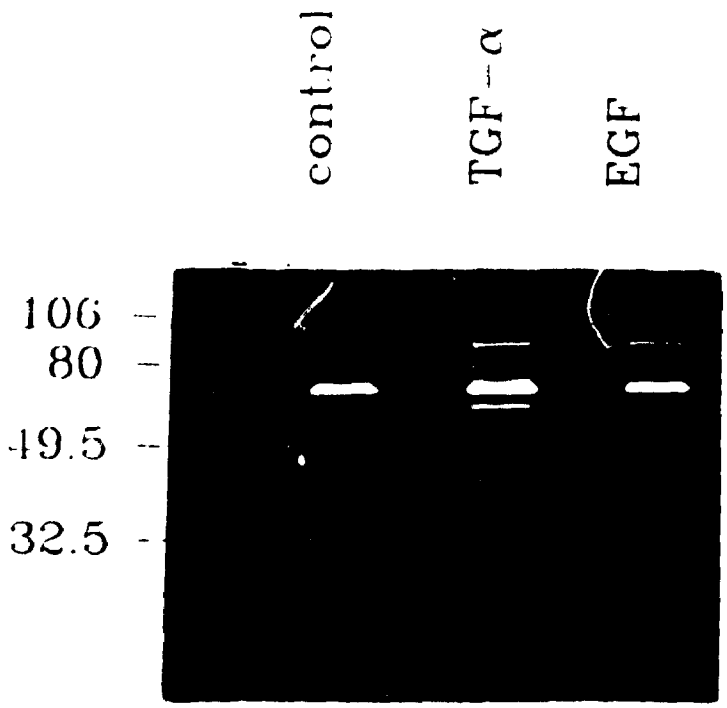


Figure 37: Gelatin zymography to examine the effects of EGF and TGFα on the production of the 72 kDa and 92 kDa type IV collagenase enzymes (gelatinase A and B respectively). Untreated first trimester trophoblast cells demonstrate gelatinase activity at the molecular weight corresponding to the 72 kDa type IV collagenase enzyme. Cells treated with EGF (10 ng/ml) or TGFα (10 ng/ml) show gelatinase activity at the molecular weights corresponding to the 72 kDa type IV collagenase as well as the 92 kDa type IV collagenase enzyme.


control
TGF−α
EGF
106
80
49.5
32.5

7. THE EFFECTS OF EGF AND TGFα ON GELATINASE ACTIVITY OF FIRST TRIMESTER HUMAN TROPHOBLAST CELLS

Results from gelatin zymography are shown in figure 37. Untreated cells (control) displayed gelatinolytic activity corresponding to the 72 kDa type IV collagenase (gelatinase A). Cells treated with EGF or TGFα demonstrated gelatinolytic activity corresponding to the 72 kDa type IV collagenase as well as activity corresponding to the 92 kDa type IV collagenase (gelatinase B). Thus, first trimester human trophoblast cells are capable of producing both the 72 kDa as well as the 92 kDa type IV collagenase enzymes in response to EGF and TGFα.

8. CHORIOCARCINOMAS

To determine if malignant human trophoblastic tumor cells respond in a similar manner as normal human first trimester trophoblast cells to the growth factors, three human choriocarcinoma cell lines, JAR, JEG3, and BeWo, were examined for their proliferative, invasive, and hCG producing responses to the growth factors.

8.1. The Effects of EGF, TGFα, and CSF-1 on the Proliferation of the Choriocarcinoma Cell Lines JAR, JEG3, and BeWo

The human choriocarcinoma cell line JAR did not show any significant alteration in proliferation in the presence of the exogenous growth factors added at a concentration

Figure 38: Effects of TGF-α, EGF, CSF-1 (all at 10 ng/ml), and anti-TGF-α neutralizing Ab (25 μg/ml) on the proliferation of the human choriocarcinoma cell line JAR. A slight increase in JAR cell proliferation ($p<0.0$[illegible] is seen in the presence of anti-TGF-α Ab. Bars represent the mean ($\pm$ SE) of quadruplicate samples. * significantly different from control values.

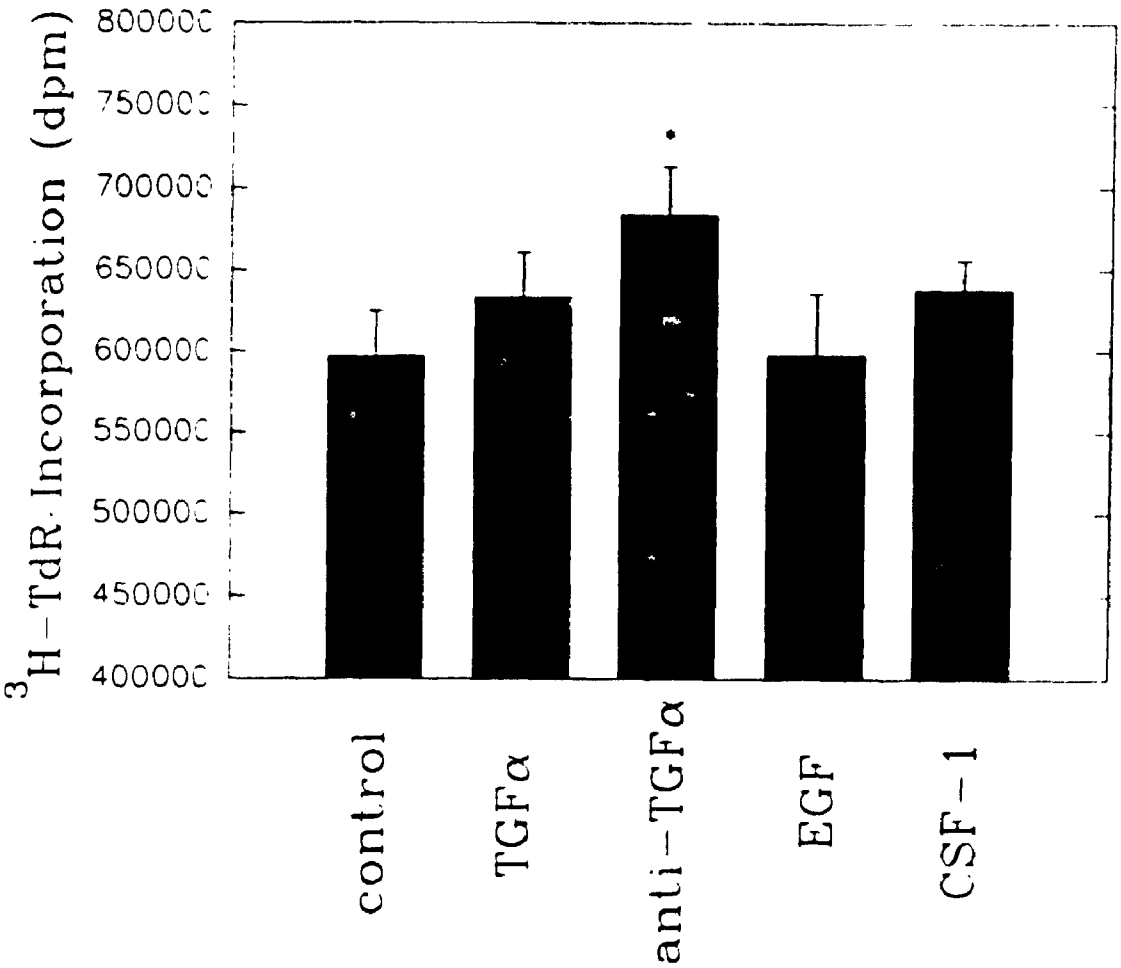
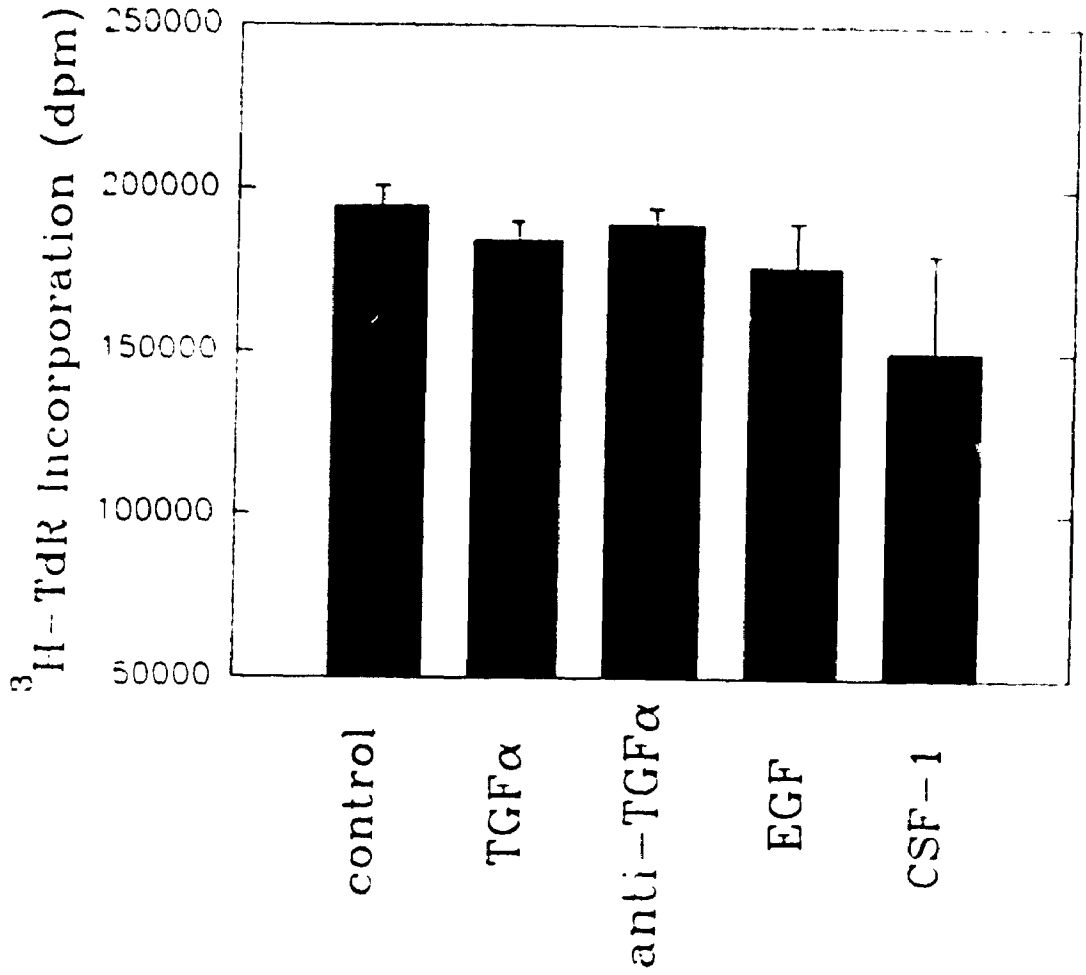

^{3}H-TdR Incorporation (dpm)
800000
750000
700000
650000
600000
550000
500000
450000
400000
control
TGFα
anti-TGFα
EGF
CSF-1

Figure 39: Effects of TGF-α, EGF, CSF-1 (all at 10 ng/ml), and anti-TGF-α neutralizing Ab (25 μg/ml) on the ^{3}H-TdR incorporation of the human choriocarcinoma cell line JEG3. No significant effect was seen on JEG3 cell proliferation with any of the treatments. Bars represent the mean ($\pm$ SE) of quadruplicate samples.


^{3}H-TdR Incorporation (dpm)
250000
200000
150000
100000
50000
control
TGFα
anti-TGFα
EGF
CSF-1

of 10 ng/ml. However, a slight increase in proliferation was observed in the presence of 25 μg/ml anti-TGFα neutralizing Ab, suggesting endogenous TGFα may have a small growth inhibitory effect (Figure 38).

JEG3 choriocarcinoma cells exhibited no significant change in proliferation in the presence of the exogenous growth factors or anti-TGFα Ab (Figure 39).

The proliferation of the human choriocarcinoma cell line BeWo was also unaffected by the addition of the exogenous growth factors or anti-TGFα Ab (Figure 40).

8.2. The Effect of TGFα on the Invasiveness of the Human Choriocarcinomas, JAR and JEG3

Results from a 3 day matrigel invasion assay are seen in figure 41. As can be seen in figure 41 the two choriocarcinoma cell lines are as invasive as normal first trimester human trophoblast cells. As already stated and observed here again exogenous TGFα or neutralizing anti-TGFα Ab did not significantly effect the invasiveness of normal first trimester human trophoblast cells. Exogenous TGFα however, caused a small but significant ($p < 0.01$) increase in the invasiveness of the choriocarcinoma cell line JAR. Neutralizing anti-TGFα Ab (25 μg/ml) did not alter JAR cell invasion, suggesting that the dose of the Ab may not have been enough to neutralize all of the endogenous TGFα. Exogenous TGFα had no significant effect on the invasiveness of JEG3 cells; however, neutralizing anti-TGFα Ab did significantly ($p < 0.05$) reduce the level of JEG3 cell invasion, perhaps suggesting that endogenous TGFα stimulates the invasiveness of JEG3 cells.

Figure 40: Effects of TGF-α, EGF, CSF-1 (all at 10 ng/ml), and anti-TGF-α neutralizing Ab (25 μg/ml) on the proliferation of the human choriocarcinoma cell line BeWo. No significant effect was seen on BeWo cell proliferation with any of the treatments. Bars represent the mean ($\pm$ SE) of quadruplicate samples.

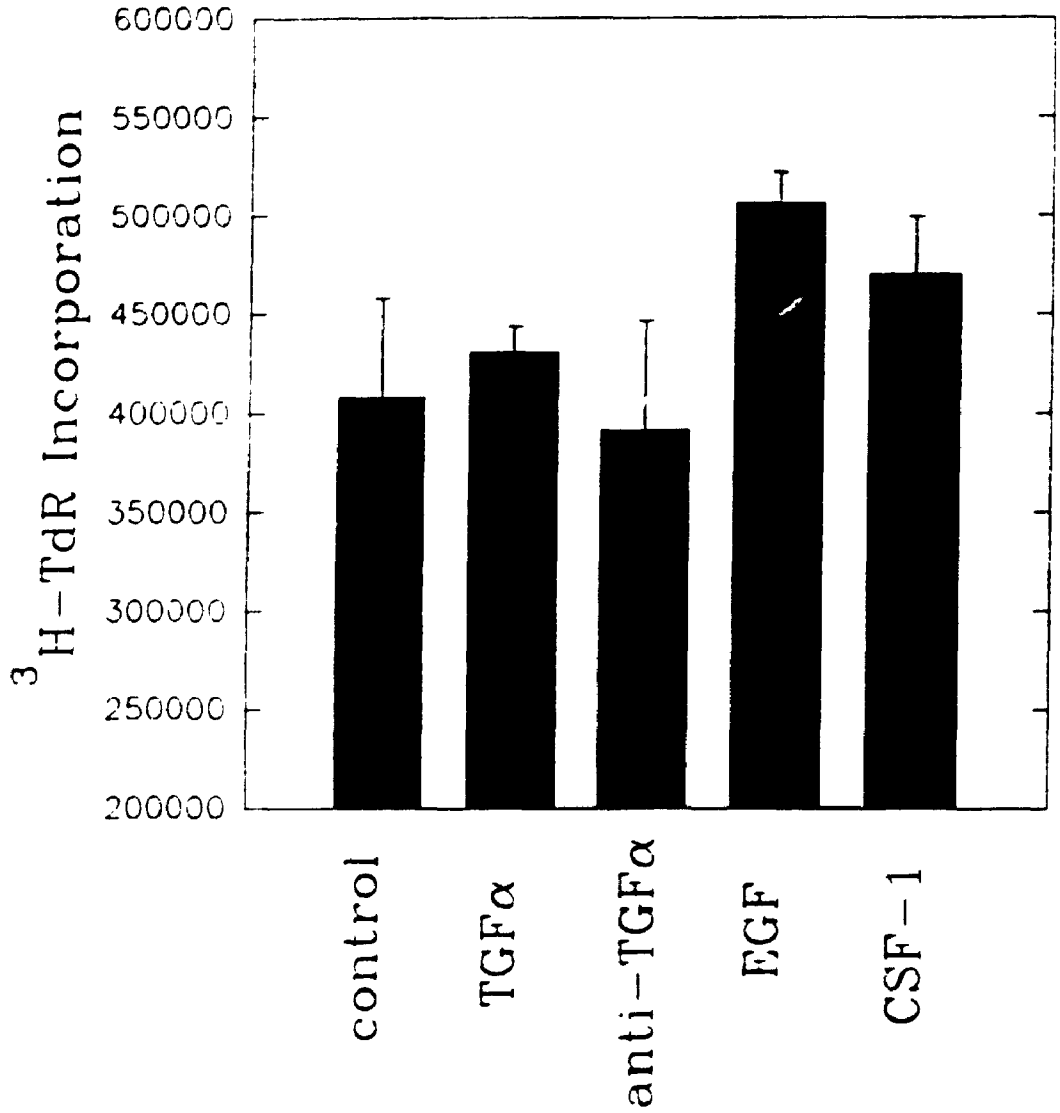

600000
550000
500000
450000
400000
350000
300000
250000
200000
^{3}H-TdR Incorporation
control
TGFα
anti-TGFα
EGF
CSF-1

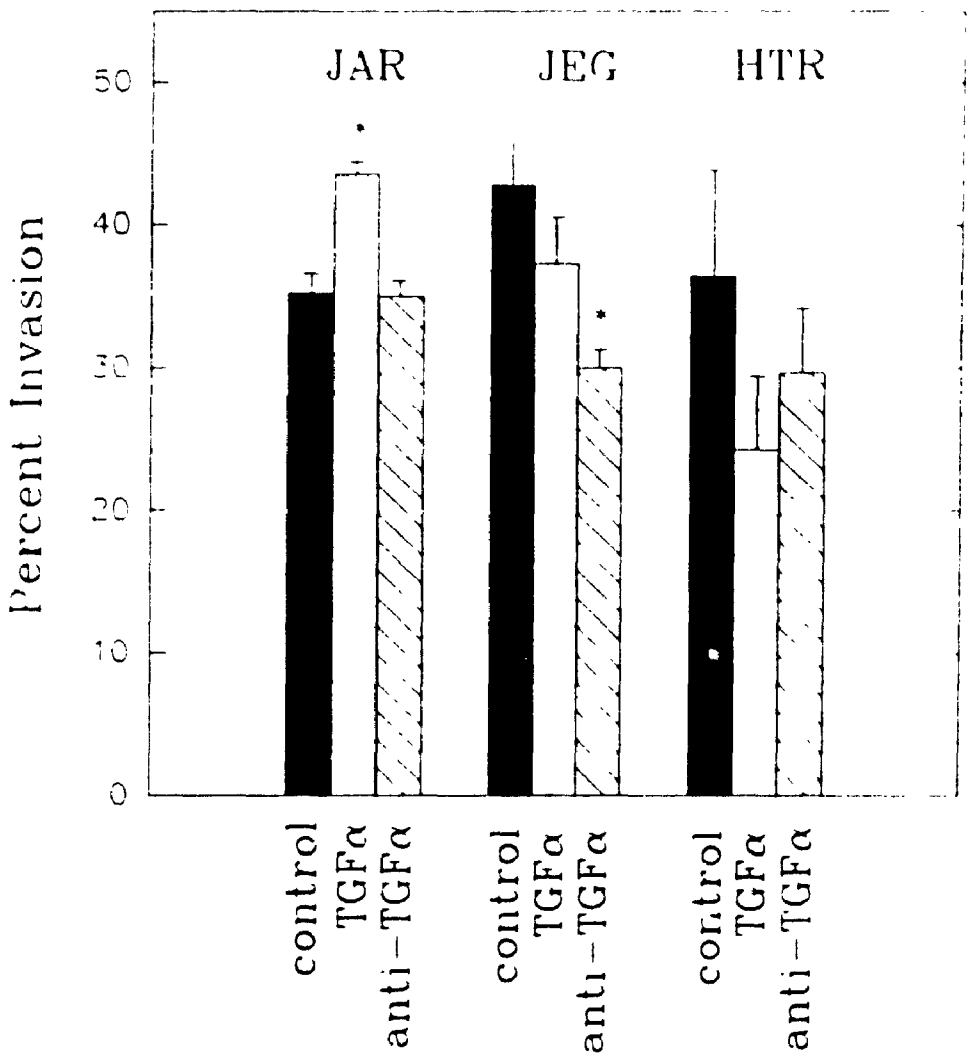
Figure 41: Results of a 3 day matrigel invasion assay with normal first trimester human trophoblast cells (HTR) and the choriocarcinomas JAR and JEG3, treated with either TGF-α (10 ng/ml), anti-TGF-α neutralizing Ab (25 μg/ml), or no additive (control). TGF-α or its Ab had no significant effect on the invasiveness on normal trophoblast cells. An increase in invasion is seen with JAR cells treated with TGF-α. JEG3 cells treated with anti-TGF-α neutralizing Ab showed a significant decrease in invasiveness. Bars represent the mean ($\pm$ SE) of triplicate samples. * significantly different from control values.

Figure 42: Northern blot analysis of the human choriocarcinoma cell line, JAR, incubated with TGF-α, EGF, CSF-1 (all at 10 ng/ml), anti-TGF-α neutralizing Ab (25 μg/ml), and no additive (control).

a) mRNA levels for the 72 kDa type IV collagenase were unaffected by any of the treatments.

b) no TIMP-1 mRNA was detected in JAR cells under any of the conditions; however, TIMP-1 mRNA was detected in HT1080 cells, used here as a control, indicating that the technique was working.

c) mRNA levels for TIMP-2 remained unaffected by any of the treatments.


JAR
JEG
HTR
Percent Invasion
50
40
30
20
10
0
control
TGFα
anti-TGFα
control
TGFα
anti-TGFα
control
TGFα
anti-TGFα

8.3. The Effects of EGF, TGFα, anti-TGFα Ab, and CSF-1 on the mRNA Levels of the Invasion Regulating Molecules (72 and 92 kDa type IV collagenases and TIMP-1 and TIMP-2) in the Choriocarcinoma Cell Line JAR

The effects of the addition of 10 ng/ml EGF, TGFα, CSF-1, and 25 μg/ml anti-TGFα neutralizing Ab on the mRNA levels of the 72 and 92 kDa type IV collagenase and TIMP-1 and TIMP-2 were assessed by Northern blot analysis. As in the case of the normal first trimester trophoblast cells no mRNA for the 92 kDa type IV collagenase enzyme was detected by Northern analysis (data not shown) of total RNA. mRNA for the 72 kDa type IV collagenase enzyme was detectable, however, it remained unaffected by the treatments (Figure 42a). Interestingly, no message for TIMP-1 was detectable in the control (no additive) or under any of the treatments (Figure 42b). TIMP-1 mRNA was observed in an internal positive control lane of RNA from the cell line HT1080, a fibrosarcoma demonstrating that the probe and the conditions were working. This same blot was also reprobed for the presence of other mRNA species indicating the presence of RNA in all of the lanes. Both transcripts, the 3.5 kb and the 0.9 kb, of the TIMP-2 molecule were detected in JAR cell RNA and they remained unchanged after any of the treatments (Figure 42c).

8.4. The Effects of EGF, TGFα, anti-TGFα Ab, and CSF-1 on Human Chorionic Gonadotrophin (hCG) Production by JAR and JEG3 Choriocarcinoma Cells

The two human choriocarcinoma cell lines, JAR and JEG3, as well as third

Figure 42: Northern blot analysis of the human choriocarcinoma cell line, JAR, incubated with TGF-α, EGF, CSF-1 (all at 10 ng/ml), anti-TGF-α neutralizing Ab (25 μg/ml), and no additive (control).

a) mRNA levels for the 72 kDa type IV collagenase were unaffected by any of the treatments.

b) no TIMP-1 mRNA was detected in JAR cells under any of the conditions; however, TIMP-1 mRNA was detected in HT1080 cells, used here as a control, indicating that the technique was working.

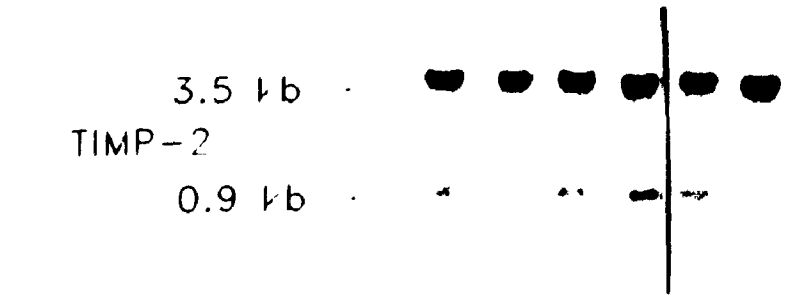
c) mRNA levels for TIMP-2 remained unaffected by any of the treatments.

Human Choriocarcinoma – JAR

A.

B.

C.



passage normal human first trimester trophoblast cells were cultured in the presence of EGF, TGFα, anti-TGFα neutralizing Ab, and CSF-1 and the conditioned media assayed for the production of hCG. Third passage normal first trimester human trophoblast (HTR-8) failed to produce any significant detectable levels of hCG in control and treated cultures (data not shown). Incubation of JAR and JEG3 cells with EGF or TGFα caused a slight nonsignificant increase in the amount of detectable hCG in the conditioned media (Figures 43 and 44).

Figure 43: Effects of TGF-α, EGF, CSF-1 (all at 10 ng/ml), anti-TGF-α neutralizing Ab (25 μg/ml), and no additive (control) on hCG production by the choriocarcinoma cell line JAR. Incubation of JAR with either TGF-α or EGF caused an increase in hCG production. Bars represent the range of duplicate samples.

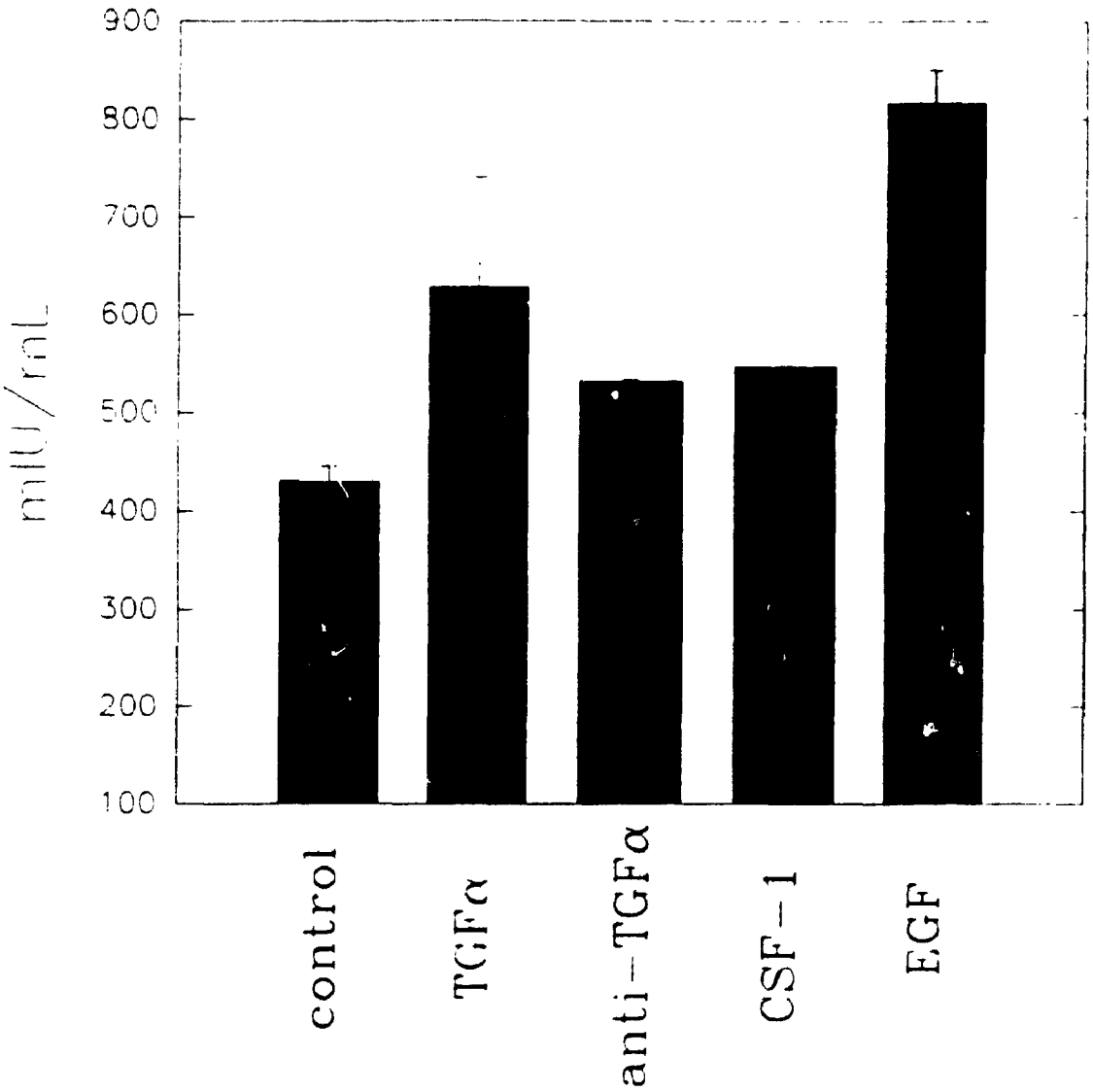
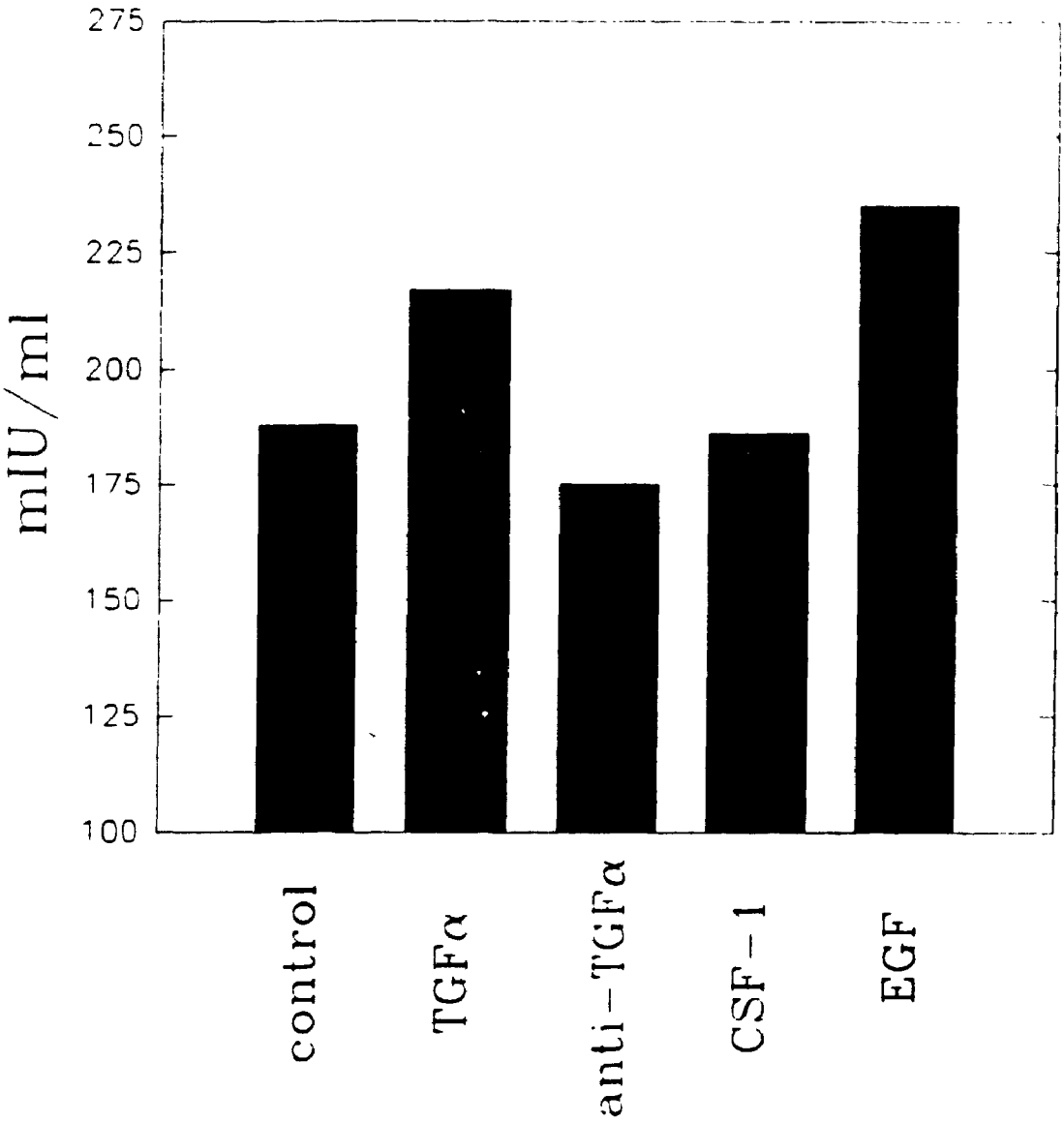

900
800
700
600
500
400
300
200
100
mIU/mL
control
TGFα
anti-TGFα
CSF-1
EGF

Figure 44: Effects of TGF-α, EGF, CSF-1 (all at 10 ng/ml), anti-TGF-α neutralizing Ab (25 μg/ml), and no additive (control) on hCG production by the choriocarcinoma cell line JEG3. Incubation of JEG3 cells with either TGF-α or EGF caused an apparent increase in hCG production. The values represent single determinations.


275
250
225
200
175
150
125
100
mIU/ml
control
TGFα
anti-TGFα
CSF-1
EGF

VI DISCUSSION

Results from the present study have demonstrated that:

(1) Mononuclear trophoblast cells migrating out of primary explants of first trimester chorionic villus fragments grown on type IV collagen gel display similar characteristics as invasive trophoblast in vivo.

Routine and immuno-electron microscopic observations of migrant cells from chorionic villus explants plated on type IV collagen gel revealed hPL positive multinucleate, syncytiotrophoblast-like, cells which appeared to be non-invasive (not penetrating the collagen gel). Mononucleate migrant cells were highly invasive cells, which penetrated the collagen gel and resided in neatly circumscribed cavities. Invasive cells also had direct focal contacts with the gel matrix. These focal contacts could possibly represent integrin mediated cell binding to type IV collagen or possibly other cell-matrix interactions. The cavities surrounding the cells could possibly be the result of enzymatic degradation of the matrix by the cell, since it is well known that invasive trophoblast cells express matrix degradative enzymes (Yagel et al., 1988; Graham and Lala, 1991, 1992;Fernandez et al., 1992; Fisher et al., 1985). The cavities may have also been formed or their width expanded by shrinkage of the cell or collagen gel retraction, or both, during fixation. These cells also possessed desmosomes, and were positive for cytokeratin thus confirming their epithelial origin. Mononucleate cells were also found to secrete both fibronectin as well as oncofetal fibronectin; however, only

intracellular labelling of fibronectin was seen. The lack of intracellular labelling for oncofetal fibronectin may be due to the possibility that the epitope is recognized by the antibody only in the extracellular, secreted form. These findings are consistent with previous work by Feinberg et al (1991) who demonstrated that isolated cytotrophoblast cells in primary culture synthesized and secreted oncofetal fibronectin. Oncofetal fibronectin was also found to be deposited at cytotrophoblast cell-ECM contact sites when cells were plated on an ECM gel (Feinberg et al., 1991). These in vitro findings correspond to in vivo observations that oncofetal fibronectin is localized to the ECM connecting extravillous trophoblast cell columns to the uterine decidua (Feinberg et al., 1991).

It is highly likely that a number of fibronectin isoforms made by the migratory trophoblast is an important part of the ECM component required for trophoblast migration. Indeed fibronectin receptors ($\alpha_5\beta_1$ integrins) have been identified on the migrant trophoblast cells in explant cultures (Irving et al., 1993).

These observations of migrant, invasive trophoblast cells from first trimester chorionic villi grown on type IV collagen are also supported by the work of Genbacev et al (1992). Who found that trophoblast cells migrated from first trimester chorionic villi plated on matrigel. These migrant cells were hPL and PCNA immunoreactive suggesting their trophoblastic origin as well as their proliferative ability. In the present study hPL immunoreactivity at the EM level was only noted in the multinucleate cells indicating that they are equivalents of the placental bed giant cells in situ. Recently, in our laboratory Irving et al (1993) have performed extensive phenotyping of these migrant trophoblast cells growing out of chorionic villus explant cultures showing that they

belong to the intermediate (extravillous) trophoblast cell population is situ.

(2) First trimester human trophoblast cells propagated in vitro have a phenotype similar to invasive intermediate trophoblast cells in vivo.

First trimester human trophoblast cells propagated out of primary explants by in vitro passage were further characterized for two trophoblast-specific markers (NDOG5 and hPL) and a marker of DNA synthesis (PCNA). Passaged trophoblast cells expressed both NDOG5 (mononucleate cells) and hPL (multinucleated cells only) and a minority of mononucleate cells expressed PCNA indicating they were in cell cycle. NDOG5 expression is seen only in the invasive extravillous trophoblast cells located within the decidua (Shorter et al., 1993). Thus, cells maintained and grown in vitro retain characteristics of extravillous trophoblast cells in vivo. It is interesting that only the multinucleated cells in vitro were immunoreactive for hPL. Placental bed giant cells, which are large multinucleated trophoblast cells in the decidua thought to be derived from the fusion of invasive extravillous trophoblast cells, also express hPL (Loke 1988; Aplin, 1991). These observations taken together suggest that the trophoblast cells maintained in culture are the invasive extravillous trophoblast cells. Further studies with these cells in our laboratory have shown that they express IGF-II protein and mRNA, class I HLA framework as recognized by W6/32 Ab, as well as the integrins subunits α_1, α_3, α_5, α_v, $ß_1$ and the vitronectin receptor complex ($\alpha_v ß_3/ß_5$; Irving et al., 1993), markers corresponding to the invasive extravillous cytotrophoblast in situ making contact with the decidua (Damsky et al., 1992; see Table 1).

(3) TGFß and its natural inhibitor, decorin, are present in the human decidua and placenta, and are colocalized in the decidual ECM.

Immunoreactive TGFß was detected in the cytoplasm of the villous syncytiotrophoblast cell layer throughout gestation. Villous cytotrophoblast cells were negative at all gestational ages; whereas, extravillous cytotrophoblast cells of the cytotrophoblastic shell showed strong cytoplasmic staining. First trimester decidual tissue showed intense TGFß immunoreactivity in the ECM with few decidual cells showing cytoplasmic staining. Decidual leukocytes and glandular epithelium were negative. At later gestational ages, the frequency of decidual cells with cytoplasmic staining increased and the staining seen in the ECM declined. At term, the majority of decidual cells showed cytoplasmic staining and the ECM was negative. These observations extend those of Graham et al (1992) from this laboratory showing that TGFß had a selective localization within the placenta. In the decidua TGFß was mostly confined to the ECM during the first trimester, whereas at term it was mostly intracellular in location. This can be explained by a higher rate of synthesis and/or a slower rate of release of TGFß by term decidual cells, or possibly by a change in TGFß binding to one or more ECM components. Graham and Lala (1991) have shown that decidua-derived TGFß is released in its inactive form, whereas TGFß released by trophoblast cells grown in culture is in its bioactive form. It is highly likely that decidua-derived TGFß is activated by the trophoblast-derived proteases eg plasmin.

Previous work from this laboratory has shown that TGFß has a number of important roles at the human fetomaternal interface, eg anti-invasive (Graham and Lala,

1991, 1992), anti-proliferative, and differentiation promoting (Graham et al., 1992). Thus, TGFß found associated the maternal decidua throughout gestation may provide a molecular barrier to confine trophoblast invasion of the uterus.

Decorin is a chondroitin-dermatan sulfate proteoglycan which has been shown to be a natural inhibitor of TGFß (Yamaguchi et al., 1990; Borders et al., 1992). Immunolocalization of decorin throughout pregnancy revealed intense immunostaining of the first trimester decidual ECM which declined towards term and immunostaining of the villous core throughout gestation. Although no intracellular staining was seen in first trimester decidual cells, they are most likely the source of decorin. This lack of intracelliular immunoreactivity may be due to either a high rate of release of decorin by decidual cells or that the intracellular form was not recognized by the antibody used. A strong co-localization of decorin and TGFß in the ECM of first trimester decidua may indicate that decorin binding facilitates TGFß storage in the ECM. Since binding to decorin is kno\ to inhibit TGFß activity it is highly likely that TGFß stored in the decidual ECM remains inactive. It is also possible that certain proteases secreted by invasive trophoblast cells are capable of releasing active form of TGFß from the decidual ECM. We offer the following hypothesis. The high degree of trophoblast invasion required during the first trimester is facilitated by the decorin inactivation of TGFß in the ECM. However, excessive invasion may be down-regulated by the activation of TGFß by trophoblast derived proteases. This hypothesis is currently being tested.

(4) AR is present in early gestational chorionic villi.

Specific AR immunoreactivity was localized to the nucleus as well as the cytoplasm of the terminally differentiated syncytiotrophoblast cell layer of chorionic villi in early gestation until approximately week 18, after which time no staining was detectable. Other trophoblast cells, viz villous cytotrophoblast and extravillous trophoblast cells, as well as decidual cells from all gestational ages examined showed no immunoreactivity. This unique spatial and temporal distribution of AR in the human placenta may be of biological significance early in human pregnancy.

Immunolocalization of AR to terminally differentiated cells has previously been reported. Johnson et al (1991, 1992), have demonstrated AR immunoreactivity in the non-proliferative terminally differentiated cells of the normal human colon. They suggest that it may play a paracrine growth stimulatory role or is involved in the differentiation of colonic epithelial cells. The significance of the nuclear location of AR remains to be determined.

The functional implications of AR localization in the placenta are discussed below.

(5) The EGF-receptor ligands, EGF, TGFα, and AR all stimulated first trimester human trophoblast proliferation; however, EGF and TGFα did not influence trophoblast invasion.

As summarized earlier (historical review) both EGF and TGFα are abundantly

produced at the fetomaternal interface, by both trophoblast and decidual cells. Our previous studies (Lysiak et al., 1993) have immunolocalized TGFα in the villous and extravillous trophoblast as well as decidual cells throughout human gestation. The presence of EGF receptors have also been shown on human trophoblast cells in vitro (Mirlesse et al., 1990; Filla et al., 1993) and in vivo (Maruo et al., 1987; Chen et al., 1988; Muhlhauser et al., 1993) indicating that EGF receptor ligands produced locally may regulate trophoblast function.

The effects of endogenous or exogenous growth factors on the proliferation of early passage first trimester trophoblast cells were measured from ^{3}H-TdR incorporation by cells exposed to ^{3}H-TdR during the last 6 hours of 24 hour culture in the presence of specific growth factors or their neutralizing antibodies. All EGF-receptor ligands, EGF, TGFα, and AR stimulated ^{3}H-TdR uptake in trophoblast cells. These results have recently been corroborated by Filla et a. (1993).

Immunohistochemical staining for TGFα in situ demonstrates TGFα immunoreactivity in villous as well as extravillous trophoblast cell populations. Recently, King and Blankenship (1993) performed PCNA immunostaining on sections of macaque placentas throughout gestation. They demonstrated that numerous cytotrophoblast cells of the chorionic villi, cytotrophoblastic shell, and cytotrophoblast cell columns (intermediate trophoblast of the anchoring villus) were immunoreactive for PCNA throughout gestation. These observations, although in the macaque, correlate with the pattern of TGFα immunoreactivity in the human placenta (Lysiak et al., 1993). Since TGFα has trophoblast growth stimulatory abilities in vitro, possible production of TGFα by cytotrophoblast cells in situ may provide growth stimulatory signals. To

investigate if trophoblast cells in vitro were responding to endogenous TGFα production, in the present study, ^{3}H-TdR incorporation was also measured in the presence of a neutralizing anti-TGFα antibody. While this antibody neutralized the growth promoting effects of exogenous TGFα, the antibody alone had no effect on trophoblast proliferation. This finding may be explained in two ways: 1) trophoblast cells maintained in culture by the present methodology did not produce significantly high levels of TGFα to affect their proliferation; or 2) TGFα produced by these cells may be in a form not neutralized by the currently used antibody. The in situ immunostaining described earlier for TGFα with anti-TGFα antibody MF9 (Lysiak et al., 1993), remains to repeated with the trophoblast cells in culture. A dramatic decline in trophoblast proliferation, however, was observed when cells were cultured in the presence of an EGF-receptor blocking antibody alone. This effect was not reversed by the addition of EGF or TGFα to the cultures. Thus, proliferation of first trimester trophoblast cells maintained in vitro appears to be dependent on an autocrine production of one or more of the EGF-receptor ligands eg, EGF, TGFα, amphiregulin, cripto, or heparin-binding EGF. The use of appropriate neutralizing antibodies as well as cytokine specific immunoassays should identify the candidate ligand.

In vivo, both villous and extravillous trophoblast cells may differentiate into multinucleate cells, villous syncytiotrophoblast and placental bed giant cells respectively. The multinucleate cells formed in first trimester invasive trophoblast cell cultures are possibly the in vivo equivalents of placental bed giant cells (Graham et al., 1992). Since TGFα provides positive signals for the proliferation of first trimester trophoblast cells in culture, we tested whether TGFα influenced the incidence of multinucleate cell

formation in vitro. Cultures were treated with TGFα as well as its neutralizing antibody for 72 hours and subsequently scored for the incidence of multinucleate cells. Exogenous TGFα decreased the incidence of multinucleate cells in these cultures, while the addition of anti-TGFα neutralizing antibody had no effect on multinucleate cell formation. TGFα may either directly affect multinucleate cell formation, by inhibiting trophoblast cell fusion, or indirectly by stimulating the selective proliferation of mononuclear cells. These possibilities remain to be discriminated by determination of the absolute number of uninucleate and multinucleate cells in culture at different intervals following incubation with TGFα.

TGFα deficient mice (waved-1 mice) have recently been described by Luetteke et al (1993). The homozygous mutant TGFα deficient mice exhibited misalignment of hair follicles, displayed eye abnormalities, and were fertile. These observations suggest that other EGF receptor ligands, such as EGF, cripto, heparin-binding EGF, or possibly CSF-1, may compensate for the TGFα loss in the placenta.

While the precise functional significance of a spatial and temporal restriction of AR localization in the early gestational trophoblast remains unknown, it may be suggested that AR may provide an additional paracrine stimulus for extensive trophoblast proliferation needed during early gestation. Nuclear localization of AR in the syncytiotrophoblast may result from nuclear binding sites with other functions.

Are trophoblast proliferation and invasion linked biological events? This question was raised because the proliferative as well as invasive functions were both inhibited by TGFß (Graham et al., 1992; Graham and Lala, 1991). Since all EGF receptor ligands stimulated trophoblast growth, whether EGF and TGFα also promoted trophoblast

invasion in vitro was also examined. Neither of these molecules had any effect on first trimester trophoblast invasion, when tested at doses which caused significant stimulation of proliferation. Northern analysis of 72 kDa type IV collagenase and TIMP-1 mRNA revealed that both mRNA's were upregulated to an approximately similar extent by EGF or TGFα and gelatin zymography revealed a stimulation in production of the 92 kDa type IV collagenase enzyme. Thus, apparently there was no net shift in the balance between the production of type IV collagenase and its inhibitor, TIMP-1, which would affect invasion. These results further support the view that trophoblast proliferation and invasion are functions which are not necessarily linked.

(6) Exogenous CSF-1 had no effect on first trimester human trophoblast cell proliferation and their invasiveness; however, endogenous CSF-1 did influence trophoblast cell proliferation.

CSF-1 mRNA (Saji et al., 1990) as well as its receptor, the *c-fms* proto-oncogene (Pampfer et al., 1992; Saji et al., 1990), have been identified in the human placenta. Immunohistochemical staining for the *c-fms* proto-oncogene has revealed that it is localized to the intermediate trophoblast cells in situ (Pampfer et al., 1992). Addition of exogenous CSF-1 to trophoblast cell cultures in vitro had no effect on cell growth; however, the addition of neutralizing CSF-1 Ab was capable of reducing trophoblast proliferation which could be rescued with exogenous CSF-1. This finding suggests that endogenous CSF-1 is an autocrine stimulant of trophoblast cell growth. Exogenous CSF-1 also had no effect on trophoblast cell invasiveness even though an upregulation of the

levels of mRNA for the 72 kDa type IV collagenase as well as TIMP-1 were noted. A lack of effect on trophoblast invasiveness can be explained by the fact that there was no net shift in the balance between degradative enzyme and its inhibitor. Thus, CSF-1 appears to function similar to the EGF-receptor ligands, by stimulating first trimester human trophoblast cell growth but having no effect on their invasive behaviour.

Mutant mice which lack CSF-1 peptide (op/op) have been reported by Pollard et al (1987). Interestingly these mice exhibit normal fertility, suggesting that other molecules important for trophoblast growth (possibly EGF receptor ligands) compensate for this deficiency.

(7) IGF-II had no effect on first trimester human trophoblast cell proliferation, but did significantly stimulate trophoblast cell invasiveness and addition of IGFBP-1 had a synergistic effect on trophoblast invasion.

Is the invasive property of trophoblast cells dependent on autocrine growth factor(s)? Because of the interesting observation that IGF-II MRNA is selectively expressed by the invasive intermediate trophoblast cells in situ throughout gestation (see Figure 3) and that IGFBP-1 MRNA is selectively expressed by the decidual cells adjacent to the intermediate trophoblast (see Figure 4), we investigated the role of these molecules on trophoblast invasion. Matrigel invasion by first trimester human trophoblast cells was stimulated in a dose-dependent manner with increasing doses of IGF-II, when the invasion assay was carried out in low serum (1% FCS) medium. Increased IGF-II dependent invasion was however not observed in the presence of media containing 10%

serum or no serum. These results reveal that in 10% serum there are molecules (perhaps certain IGFBPs) which abrogate the invasion stimulatory effects of IGF-II. In serum deficient media the concentration of invasion promoting IGFBP-1 (see discussion later) may not have been sufficient to interact with IGF-II or enough plasminogen may not have been present to be activated to plasmin and thus in turn activate the type IV collagenases.

The IGF-II induced stimulation of trophoblast invasion in low serum containing media was further enhanced in a synergistic manner in the presence of IGFBP-1, even at minute (0.1 nM) doses. This synergy may have resulted from an increased affinity or stability of IGF-II binding to its receptor when complexed with IGFBP-1. The latter is a likely possibility, since IGFBP-1 contains an RGD sequence (reviewed by Shimasaki and Ling, 1991) which can bind to RGD binding sites on the cell surface. Such binding sites are known to be a part of cell surface integrin molecules. Indeed, it has recently been shown in our laboratory that IGFBP-1 stimulates trophoblast migration in vitro, which can be blocked by pretreatment of the cells with anti-α_5 or anti-β_1 integrin antibodies (Irving and Lala, 1994).

The mechanisms of the IGF-II invasive stimulatory effects on trophoblast cells remain to be determined by measuring the mRNA levels of invasion promoting proteases eg type IV collagenase (72 and 92 kDa) and uPA as well as their inhibitors TIMP-1, TIMP-2, PAI-1 and PAI-II. An upregulation of type IV collagenase remains a strong possibility. Recently, Blankenship and King (1994) performed immunohistochemistry for the 72 kDa type IV collagenase in the macaque placenta and found that extravillous cytotrophoblast cells embedded in the decidua as well as endovascular trophoblast cells were strongly immunoreactive for this enzyme.

The nature of IGF receptor(s) on the trophoblast cells responsible for the invasion promoting effects of IGF-II remains unidentified, and are currently under study in this laboratory. The finding that IGF-II stimulates trophoblast invasion without influencing proliferation further substantiates the view that the two processes are not linked.

Heterozygous mice carrying a disrupted IGF-II gene have been reported to be smaller (60% of normal body weight) than their normal littermates. The lack of normal levels of IGF-II may have a direct affect on numerous developmental processes; however, it could not be ruled out that the growth-deficient phenotype is an indirect consequence of impaired placental functions (DeChiara et al., 1990).

The fact that IGFBP-1 alone stimulated trophoblast invasion may suggest that a binding of IGFBP-1 to the RGD binding sites on the cell surface is stabilizing endogenous IGF-II produced by trophoblast cells, and thus influencing their invasiveness, and/or is exerting a direct effect on trophoblast invasiveness independent of IGF-II. Recently Jones et al (1993) have demonstrated that IGFBP-1 alone is able to stimulate the migratory ability of Chinese hamster ovary (CHO) cells and identified the $\alpha_5\beta_1$ integrin (fibronectin receptor) as the cell surface receptor that most likely mediates this event. Recent results from this laboratory (Irving et al., 1993) have demonstrated that first trimester human trophoblast maintained in culture according to the currently used isolation procedure express the $\alpha_5\beta_1$ integrin; thus, it is possible in trophoblast cells that IGFBP-1 may bind to this integrin and stimulate their invasiveness. As mentioned earlier, IGFBP-1 alone stimulates trophoblast cell migration, and this is dependent on α_5/β_1 integrin subunits on the cell surface (Irving and Lala, 1994). Whether IGFBP-1 also influences trophoblast cell proliferation remains to be investigated.

(8) The human choriocarcinoma cell lines examined showed alterations in their responses to the addition of growth factors, compared to normal first trimester trophoblast cells.

Experiments conducted to examine the effect of TGFα, EGF, and CSF-1 on hCG production revealed that the early passage normal trophoblast cells failed to produce significant levels of hCG, whereas, both JAR and JEG3 cells showed an increased hCG production in response of TGFα or EGF. Previous studies have reported stimulatory effects of EGF on hCG production in primary cultures of first trimester trophoblast (Maruo et al., 1987; Morrish et al., 1987) as well as choriocarcinoma cells (Huot et al., 1981). Present results of the loss of hCG producing ability of first trimester trophoblast cells propagated by the current method is in agreement with earlier reports from this laboratory (Graham et al., 1992) and suggest that a hCG-nonproducing subset of trophoblast cells is selectively expanded in the present method of propagation. Since extravillous invasive trophoblast cells in situ show no evidence of hCG production, we interpret our data as additional evidence in support of the hypothesis that these cells are the in vivo equivalents of the invasive extravillous trophoblast.

The proliferation of the human choriocarcinoma cell lines, JAR, JEG3, and BeWo, was not affected by the addition of exogenous TGFα, EGF, or CSF-1. Addition of neutralizing anti-TGFα Ab to JAR cells resulted in a slight increase in proliferation suggesting that endogenous TGFα may be growth inhibitory for JAR cells. This is a surprising finding which requires to be confirmed in further experiments. The effect of TGFα was also investigated on the invasiveness of JAR and JEG3 cells. TGFα stimulated the invasiveness of JAR cells while the addition of anti-TGFα neutralizing Ab

decreased the invasiveness of JEG3 cells suggesting in both cases that TGFα promotes their matrigel invasiveness. The differential response of the choriocarcinoma cell lines to the growth factors as compared to normal first trimester human trophoblast cells may have resulted from the genetic changes during their malignant transformation in vivo, or further genetic changes during their propagation in culture for many generations. Indeed, recent studies from this laboratory reveaied that JAR and JEG3 choriocarcinoma cells are resistant to both antiproliferative as well as anti-invasive signals provided by TGFß (Graham et al., 1993), a finding which may explain uncontrolled proliferation and invasion in both.

Results from the studies described in this thesis provide further evidence that the human placenta shares some properties with invasive tumors, however placental invasion remains highly regulated. Cellular invasion, angiogenesis, and evasion of the host's immune system are some of the properties the placenta shares with tumors. What makes an invasive cell tumorgenic? What makes a tumorgenic cell metastatic? These are some of the questions which are study in this laboratory using placental trophoblast as a model system in vivo.

VII CONCLUSIONS

This study has demonstrated that a number of growth factors present at the human fetomaternal interface influence trophoblast growth and invasion in an autocrine or paracrine manner.

EGF receptor ligands are shown to be an important family of growth-stimulatory factors for the human placental trophoblast, in vitro, and therefore possibly also in vivo. Some of these ligands eg EGF and TGFα are produced by both the trophoblast and the decidua and thus function in both autocrine and paracrine modes; whereas, AR, the location of which is restricted to the syncytiotrophoblast, must function in a paracrine mode since syncytiotrophoblast cells are themselves nonproliferative. CSF-1, known be an important product of glandular epithelium, as well as the placental trophoblast, is another possible promoter of trophoblast growth. Spatial and/or temporal selectivity in trophoblast proliferation during placental development is possibly determined by the different availability of these growth promoting molecules as well as their receptors on trophoblast cell subsets. Finally, negative regulation must also operate in a similar manner in controlling trophoblast growth.

None of the above growth stimulatory factors (EGF receptor ligands and CSF-1) influenced trophoblast invasiveness. IGF-II, shown to be produced by invasive trophoblast cells in vivo, had no effect on trophoblast cell growth in vitro, but did significantly enhance trophoblast cell invasion in vitro suggesting that IGF-II may regulate trophoblast cell invasiveness in vivo in an autocrine manner. Thus, trophoblast cell proliferation and invasion are two independent events influenced by different growth

factors and are not necessarily linked functions.

IGFBP-1 alone stimulated trophoblast cell invasiveness in vitro and in combination with IGF-II greatly synergised this stimulatory effect. IGFBP-1 has been shown to be produced by the decidual cells in the vicinity of the invasive trophoblast cell in vivo. Thus this factor may act in a paracrine or juxtacrine manner on the invasive trophoblast. Binding of IGFBP-1 to the trophoblast by the RGD binding site may directly stimulate the invasive mechanisms eg cell migration; it may also stabilize the binding of IGF-II to its receptor(s) on the trophoblast, thus synergising IGF-II action.

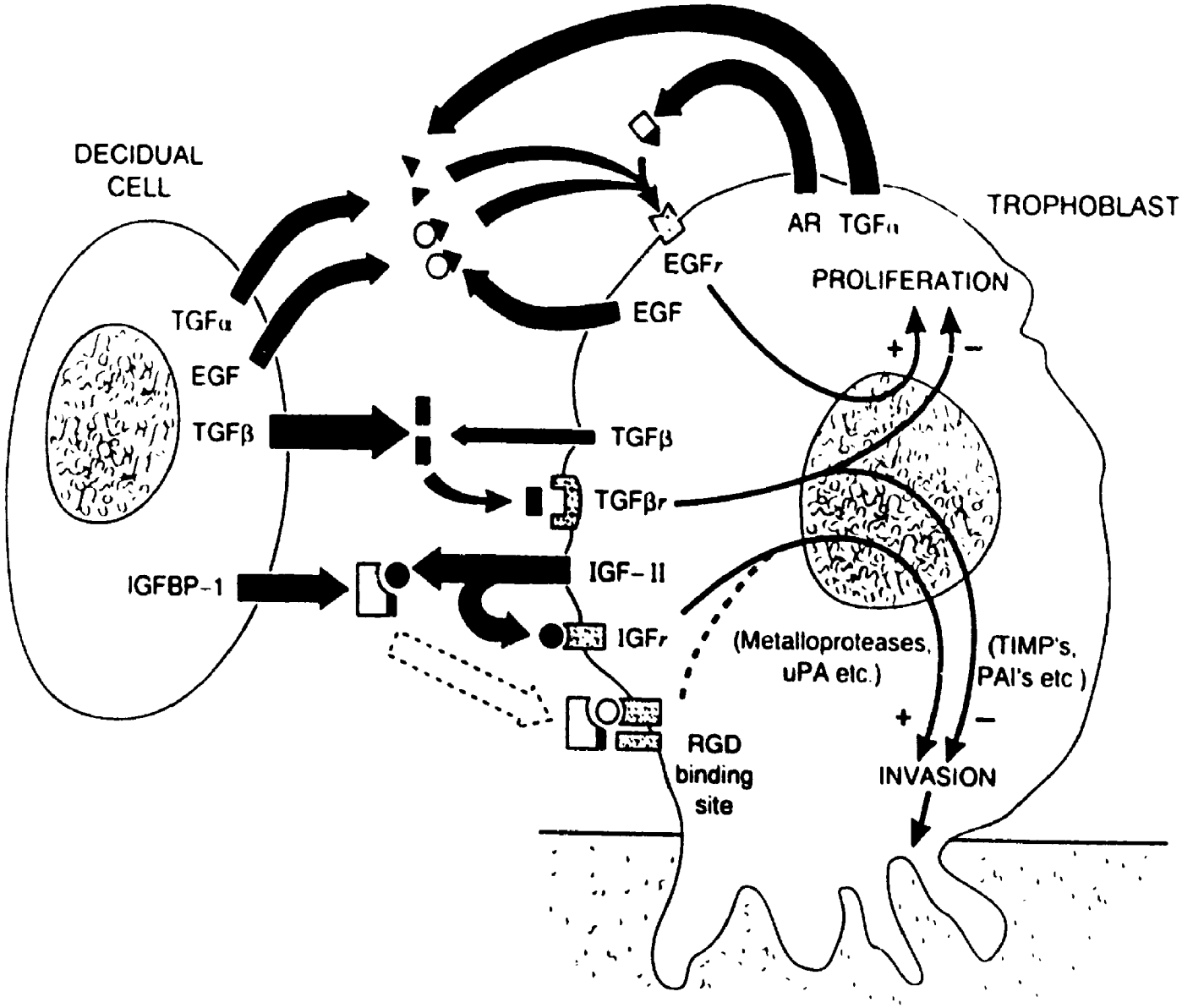
The findings that decidual cells at the human fetomaternal interface produce both invasion inhibitory molecules eg TGFß and invasion promoting molecules eg IGFBP-1 may be puzzling. This also applies to the fact that decidual cells can produce growth inhibitory molecules eg TGFß and growth stimulatory molecules eg TGFα and EGF. Thus it appears that decidua may have a broad regulatory role for placental growth and invasion. It is highly likely that the regulatory functions in situ are highly discriminatory in nature so that different decidual cells may be responsible for the production of up or down-regulatory molecules. For example, only a small subset of decidual cells in the first trimester decidual tissue were shown to have intracellular TGFß. Alternatively, the regulation can also be dictated by differential expression of receptors/binding sites on the trophoblast cell surface in situ. These hypotheses can be tested by double labeling in situ for regulatory factors or their receptors/binding sites.

Results presented in this thesis, combined with earlier reports from this laboratory point towards a very important general physiological principle regarding trophoblast invasion of the uterus: human trophoblast cells are genetically equipped with invasive

ability, as well as the ability to upregulate their own invasiveness in an autocrine manner. A control of this invasiveness, or its further upregulation in situ is predominantly provided by the decidua in a paracrine (or possibly juxtacrine) mode.

Figure 45 presents a scheme of autocrine/paracrine/juxtacrine interactions of the trophoblast and the decidua which may regulate trophoblast growth and invasion.

Figure 45: A schematic diagram of autocrine/paracrine regulation of trophoblast proliferation and invasion by locally produced growth factors. All EGF receptor ligands (EGF { } and TGFα { }: autocrine, paracrine; AR { }: autocrine) stimulate proliferation without affecting invasion. TGFß (: paracrine and to a small extent autocrine) downregulates proliferation as well as invasion. IGF-II (; autocrine) stimulates invasion with no affect on proliferation. IGFBP-1) synergises IGF-II action possibly by binding to RGD binding sites on the cell surface. Stimulation or inhibition of invasion results from a shift in the balance between degradative enzymes and their natural inhibitors.


DECIDUAL CELL
TROPHOBLAST
TGFα
EGF
TGFβ
IGFBP-1
AR TGFα
EGFr
EGF
PROLIFERATION
+
−
TGFβ
TGFβr
IGF-II
IGFr
(Metalloproteases, uPA etc.)
(TIMP's, PAI's etc.)
+
−
RGD binding site
INVASION

VIII QUESTIONS TO BE ANSWERED

1) Does decorin, in the ECM of first trimester decidual tissue, bind to and inhibit local TGFß activity?

If so, does the production of proteases release TGFß from decorin?

2) Does decorin influence first trimester human trophoblast cell growth and invasiveness?

3) Do trophoblast cell in culture (isolated and maintained by the currently used protocols) produce significant levels TGFα, EGF, AR, or CSF-1?

4) What type of IGF receptors are present of trophoblast cells?

5) What are the molecular mechanisms by which IGF-II stimulates trophoblast cell invasiveness?

6) What are the mechanisms by which IGFBP-1 influences trophoblast cell invasion?

7) How does IGFBP-1 interact with IGF-II to stimulate invasion by first trimester human trophoblast cells.

8) What is present in 10% FCS that inhibits the dose-dependent increase in invasion seen with IGF-II?

9) Do different decidual cells in situ produce stimulatory and inhibitory molecules which influence trophoblast growth and invasion?

IX ORIGINAL CONTRIBUTIONS

A. Characterization of Trophoblast Cells Isolated and Propagated In Vitro

1. Cells from explants grown on type IV collagen gel revealed two trophoblast cell populations containing cytokeratin: invasive mononucleate cells which produce fibronectin and oncofetal fibronectin, and noninvasive multinucleate cells which contain hPL.

2. Early passage trophoblast cells were highly invasive and positive for cytokeratin (100%), NDOG5 (50%; an intermediate trophoblast cell marker), PCNA (small proportion), and hPL (multinucleate cells only), providing further evidence that they belong to the invasive extravillous trophoblast in situ.

B. Immunolocalization of Amphiregulin, TGFß, and Decorin in the Human Placenta and the Decidua Throughout Gestation

1. AR exhibited a unique spatial and temporal distribution, being present only in the syncytiotrophoblast cell layer of the placenta until approximately week 18 of gestation, and never in the decidua. This may suggest a paracrine growth stimulatory role of AR for the cytotrophoblast cells (see later).

2. TGFß was present in the decidua as well as the villous syncytiotrophoblast and extravillous cytotrophoblast cells throughout gestation. Decorin

immunoreactivity displayed a pattern of distribution similar to the distribution of TGFß in the ECM of first trimester decidual tissue, suggesting that decorin may influence TGFß activity in first trimester decidua.

C. *The Effects of the Growth Factors on First Trimester Human Trophoblast Cell Proliferation and Invasion*

1. Exogenous EGF receptor ligands (EGF, TGFα, and AR) were able to stimulate trophoblast proliferation in a concentration-dependent manner. EGF and TGFα however did not influence trophoblast invasion even though an increase in the mRNA and protein levels of invasion regulating molecules (72 and 92 kDa type IV collagenases as well as TIMP-1 and TIMP-2) were noted.

2. While exogenous CSF-1 had no effect on trophoblast proliferation or invasion, endogenous CSF-1 appeared to upregulate proliferation as noted from the action of a neutralizing antibody.

Thus EGF receptor ligands and CSF-1 may be required for trophoblast growth in situ.

3. Exogenous IGF-II demonstrated no significant effect on trophoblast proliferation; however, it significantly enhanced the invasiveness of first trimester human trophoblast cells in a concentration-dependent manner. This stimulation was further enhanced in a synergistic manner in the presence of IGF binding

protein (IGFBP)-1. Thus autocrine derived IGF-II and paracrine derived IGFBP-1 may interact in situ to promote trophoblast invasiveness.

These studies reveal that trophoblast proliferation and invasion are independent events.

D. The Effects of the Growth Factors on Human Choriocarcinoma Cells

1. Choriocarcinomas cell lines (JAR and JEG3) were generally unresponsive to addition of growth factors (TGFα, EGF, CSF-1) with the exception of the invasion promoting effects of TGFα noted on JAR cells. These results combined with the earlier demonstration of resistance of these choriocarcinoma cells to the antiproliferative and anti-invasive action of TGFß, suggest genetic alterations are responsible for changes in their response to growth factors.

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